MECANISMOS DE POTENCIACIÓN DE LAS DEFENSAS ANTIOXIDANTES ENDÓGENAS FRENTE AL ESTRÉS OXIDATIVO

Tesis doctoral

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MECANISMOS DE POTENCIACIÓN DE LAS DEFENSAS ANTIOXIDANTES ENDÓGENAS FRENTE AL ESTRÉS OXIDATIVO

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ALA	Ácido δ-aminolevulínico / *δ-aminolevulinic acid*
ALAD	Ácido δ-aminolevulínico dehidratasa / *δ-aminolevulinic acid dehydratase*
AMP	Adenosín monofosfato (cAMP, AMP cíclico) / *Adenosine monophosphate (cAMP, cyclic AMP)*
AP-1	Proteína activadora 1 / *Activator protein 1*
ARE	Elemento de respuesta antioxidante / *Antioxidant response element*
ATP	Adenosín trifosfato / *Adenosine triphosphate*
CAT	Catalasa / *Catalase*
CDR	Cantidad diaria recomendada / *Recommended Daily Allowance*
CH	Coproporfiria hereditaria / *Hereditary coproporphyria*
CK	Creatina quinasa / *Creatine kinase*
CoQ_{10}	Coenzima Q_{10} / *Coenzyme Q_{10}*
CREB	Proteína de unión a elementos de respuesta a cAMP / *cAMP response element binding*
DAG	Diacilglicerol / *Diacylglycerol*
DNA	Ácido desoxirribonucleico / *Deoxyribonucleic acid*
ER	Receptor de estrógenos / *Estrogen receptor*
ERK	Quinasas reguladas por señales extracelulares / *Extracellular signal-regulated kinases*
GC	Guanilato ciclasa (sGC, soluble) / *Guanylate cyclase (sGC, soluble)*
GGT	Gamma-glutamil-transpeptidasa / *Gamma-glutamyl-transpeptidase*
GMP	Guanosín monoifosfato (cGMP, GMP cíclico) / *Guanosine monophosphate (cGMP, cyclic GMP)*
GDP	Guanosín bifosfato / *Guanosine diphosphate*
GTP	Guanosín trifosfato / *Guanosine triphosphate*
GPx	Glutatión peroxidasa / *Glutathione peroxidase*
GRd	Glutatión reductasa / *Glutathione reductase*
GRX	Glutaredoxina / *Glutaredoxin*
GSH	Glutatión reducido / *Reduced glutathione*
GSSG	Glutatión oxidado o glutatión disulfuro / *Oxidized glutathione or glutathione disulfide*
Hb	Hemoglobina (metHb, metahemoglobina) / *Hemoglobin (metHb, methemoglobin)*

HBO	Oxígeno hiperbárico / *Hyperbaric oxygen*
HCM	Hemoglobina corpuscular media / *Mean corpuscular hemoglobin*
4-HNE	4-hidroxinonenal / *4-hydroxynonenal*
HO-1	Hemooxigenasa-1 / *Heme oxygenase-1*
HPLC	Cromatografía líquida de alta eficacia / *High performance liquid cromatography*
HSP	Proteínas de choque térmico / *Heat shock proteins*
IKK	IκB quinasa / IκB kinase
IL	Interleuquina / *Interleukin*
JNK	Quinasas N-terminales de c-Jun / *c-Jun N-terminal kinase*
LDH	Lactato deshidrogenasa / *Lactate dehydrogenase*
MAPK	Proteína quinasa activada por mitógeno / *Mitogen-activated proteic kinase*
MDA	Malondialdehído / *Malondialdehyde*
MHC	Complejo mayor de histocompatibilidad / *Major histocompatibility complex*
MPO	Mieloperoxidasa / *Myeloperoxidase*
NAD^+/NADH	Nicotinamida-adenina dinucleótido / *Nicotinamide adenine dinucleotide*
$NADP^+$/NADPH	Nicotinamida-adenina dinucleótido fosfato / *Nicotinamide adenine dinucleotide phosphate*
NF-κB	Factor nuclear kappaB / *Nuclear factor kappaB*
NK	Células *natural killer* / *Natural killer cells*
NOS	Óxido nítrico sintasa (eNOS, endotelial; iNOS, inducible; nNOS, neuronal) / *Nitric oxide synthase (eNOS, endothelial; iNOS, inducible; nNOS, neuronal)*
8-OHdG	8-hidroxi-7,8-dihidro-2'-deoxiguanosina / 8-hydroxy-7,8-dihydro-2'-deoxyguanosine
PAI	Porfiria aguda intermitente / *Acute intermittent porphyria*
PBG	Porfobilinógeno / *Porphobilinogen*
PBG-D	Porfobilinógeno deaminasa / *Porphobilinogen deaminase*
PBS	Tampón fosfato salino / *Phosphate buffered saline*
PCT	Porfiria cutanea tarda / *Porphyria cutanea tarda*
PDA	Porfiria por deficiencia de ALA dehidratasa / *ALA dehydratase deficient porphyria*
PDGF	Factor de crecimiento derivado de plaquetas / *Platelet-derived*

	growth factor
PEC	Porfiria eritropoyética congénita / *Congenital erythropoietic porphyria*
PGC-1α	Coactivador de PPARγ 1alpha / *PPARγcoactivator 1alpha*
PI_3K	Fosfoinositol 3-quinasa / *Phoshphoinositide 3 kinase*
PKC	Proteína quinasa C / *Proteic kinase C*
PMA	12-miristato 13-acetato de forbol / *Phorbol 12-myristate 13-acetate*
PPARγ	*Peroxisome proliferator-activated receptor gamma*
PPE	Protoporfiria eritropoyética / *Erythropoietic protoporphyria*
PPOX	Protoporfirinógeno oxidasa / *Protoporphyrinogen oxidase*
PTK	Tirosina quinasa / *Protein tyrosine kinase*
PTP	Tirosina fosfatasa / *Protein tyrosine phosphatase*
PV	Porfiria variegata / *Variegate porphyria*
RFA	Respuesta inmunitaria de fase aguda / *Acute phase immune response*
RNA	Ácido ribonucleico / *Ribonucleic acid*
RNS	Especies reactivas de nitrógeno / *Reactive nitrogen species*
ROS	Especies reactivas de oxígeno / *Reactive oxygen species*
SIRT	Sirtuina / Sirtuin
SOD	Superóxido dismutasa (ecSOD, extracelular) / *Superoxide dismutase (ecSOD, extracellular)*
TAB	Tejido adiposo blanco / *White adipose tissue*
TAM	Tejido adiposo marrón / *Brown adipose tissue*
TBARS	Sustancias reactivas del ácido tiobarbitúrico / *Thiobarbituric acid reactive substances*
TCR	Receptor de linfocitos T / *T cell receptor*
TNF-α	Factor de necrosis tumoral alfa / *Tumor necrosis factor alpha*
TRX	Tioredoxina / *Thioredoxin*
UCP	Proteína desacoplante / *Uncoupling protein*
VCM	Volumen corpuscular medio / *Mean corpuscular volume*
VNTR	Repeticiones en tándem de número variable / *Variable number tandem repeats*
XDH	Xantina deshidrogenasa / *Xanthine dehydrogenase*
XO	Xantina oxidasa / *Xanthine oxidase*

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Resumen

Las especies reactivas de oxígeno son moléculas altamente reactivas que pueden reaccionar con facilidad con los diferentes componentes moleculares de las células, induciendo cambios en su estructura y alterando su función. Sin embargo, estas mismas especies reactivas pueden a su vez inducir una respuesta protectora en la célula a través de la activación de las defensas antioxidantes cuya función es eliminar estas especies reactivas o reparar moléculas oxidadas. El estado de las defensas antioxidantes previo, así como la respuesta inmediata, al desequilibrio oxidativo generado determinarán el futuro de la célula y la nueva situación de equilibro que se instaure. Así, el objetivo central de esta tesis ha sido encontrar situaciones y estrategias que potencien las defensas antioxidantes endógenas, aportando una mayor protección frente a la aparición de estrés oxidativo, y profundizar en los mecanismos implicados en esta adaptación celular.

Para alcanzar dicho objetivo hemos estudiado la repercusión de la actividad física (como una situación aguda de incremento de las especies reactivas de oxígeno) y la enfermedad porfiria variegata (como una situación hiperoxidativa crónica) sobre el daño oxidativo y las defensas antioxidantes en diferentes tipos celulares, así como la influencia del género en las adaptaciones celulares al ejercicio. Adicionalmente, hemos definido el papel de la suplementación de la dieta con nutrientes antioxidantes en el contexto de situaciones agudas y crónicas de estrés oxidativo, así como su interacción con los procesos de hormesis atribuidos a las especies reactivas de oxígeno.

La práctica de un ejercicio de alta intensidad genera un incremento en la producción linfocitaria de especies reactivas de oxígeno y en los niveles de peroxidación lipídica, a la vez que se activan las defensas antioxidantes a nivel transcripcional y post-transcripcional. El coactivador transcripcional PGC-1α y la sirtuina SIRT3 parecen ser efectores clave de la respuesta adaptativa del linfocito al ejercicio. La explosión oxidativa en el neutrófilo en respuesta a un ejercicio intenso coexiste con un incremento en la expresión de enzimas antioxidantes y una

disminución en su actividad intracelular, lo que en el caso de la catalasa se explica por una salida controlada del enzima desde el neutrófilo hasta el medio extracelular. La respuesta antioxidante al ejercicio difiere en función del género. Una sesión de actividad física intensa incrementa la producción de especies reactivas de oxígeno y de óxido nítrico, así como la expresión de iNOS y Mn-SOD, únicamente en chicos. Las mujeres afectadas de porfiria variegata presentan una situación de estrés oxidativo crónico caracterizada por niveles elevados de marcadores de daño oxidativo y en la que las defensas antioxidantes enzimáticas se encuentran activadas en eritrocitos y disminuidas en linfocitos y neutrófilos. La ingesta de una bebida de almendra suplementada con vitamina C (150 mg/día) y vitamina E (50 mg/día) potencia la actividad basal de los enzimas antioxidantes en linfocitos y eritrocitos, mediante mecanismos post-transcripcionales, a la vez que induce un descenso en los niveles de peroxidación lipídica en plasma en mujeres afectadas de porfiria variegata y en mujeres sanas. La ingesta de la misma bebida, adicionalmente enriquecida con fitoestrógenos, potencia la activación de los enzimas antioxidantes eritrocitarios en respuesta a una sesión de natación en deportistas bien entrenados. La exposición de un cultivo celular a una generación sostenida de H_2O_2 de 1 y 10 nM/s induce un incremento en la expresión génica y la actividad de las defensas antioxidantes enzimáticas, a la vez que activa la expresión del PGC-1α, si bien esta activación de las defensas antioxidantes no previene la aparición inmediata de daño oxidativo.

En conclusión, la activación de las defensas antioxidantes y la inducción de daño oxidativo ejercidas por las especies reactivas de oxígeno son dos procesos que se encuentran estrechamente relacionados, ya que las concentraciones más bajas de especies reactivas que se muestran suficientes para activar las defensas conllevan también la aparición de daño oxidativo. La ingesta de antioxidantes en dosis moderadas pero superiores a la cantidad diaria recomendada se muestra efectiva a la hora de potenciar las defensas antioxidantes endógenas y de disminuir los niveles de oxidación molecular, por lo que estos nutrientes parecen adecuados para aumentar la protección antioxidante y prevenir la aparición de daño oxidativo.

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Summary

Reactive oxygen species are highly reactive molecules which can react easily with the molecular components of cells, thus inducing both structural and functional changes. However, these reactive species can also induce an endogenous protective response in the cell through the activation of the antioxidant defenses, which in turn will scavenge the reactive species or repair the oxidized molecules. The status of the antioxidant defenses and the immediate response to the oxidative disequilibrium will determine the future of the cell and the instauration of a new situation of equilibrium. Therefore, the aim of this thesis was to establish different strategies which might enhance the endogenous antioxidant defenses and provide the cell with a higher protection against the apparition of oxidative stress, as well as to gain a better understanding on the mechanisms involved in this cellular adaptation.

We have studied the effects of physical activity (as a situation of an acute increase in reactive oxygen species) and variegate porphyria disease (as a chronic hyperoxidative situation) on oxidative damage and antioxidant defenses in different cell types, as well as the influence of gender on the cellular adaptations to exercise. We have additionally defined the effects of a diet supplementation with antioxidant nutrients on both acute and chronic situations of oxidative stress and the interaction between these nutrients and the hormetic processes attributed to reactive oxygen species.

Intense exercise induces higher reactive oxygen species production and higher levels of lipid peroxidation in lymphocytes, and the antioxidant defenses are activated both at the transcriptional and post-transcriptional levels. The transcriptional coactivator PGC-1α and sirtuin SIRT3 seem key effectors of this adaptative response to exercise in lymphocytes. The oxidative burst in neutrophils in response to intense exercise coexists with increased expression and decreased activity of the antioxidant enzymes. This behaviour can be explained, at least for catalase, through a controlled enzyme release from the neutrophil to the extracellular compartment. The antioxidant response to exercise is influenced by gender. Physical activity increases reactive oxygen species

and nitric oxide production, as well as iNOS and Mn-SOD expression, only in boys. Women affected by variegate porphyria present a situation of chronic oxidative stress, characterized by increased levels of oxidative damage markers, decreased antioxidant defenses in lymphocytes and neutrophils and increased activities of the antioxidant enzymes in erythrocytes. The intake of an almond based beverage supplemented with vitamin C (150 mg/day) and vitamin E (50 mg/day) enhances the basal activity of antioxidant enzymes in lymphocytes and erythrocytes through post-transcriptional mechanisms, while decreases the lipid peroxidation in plasma, both in women affected by variegate porphyria and control healthy women. The intake of the same beverage additionally enriched with phytoestrogens enhances the activation of the antioxidant enzymes in response to a swimming session in erythrocytes of well trained swimmers. The exposure of a cell culture to a sustained production of 1 and 10 nM H_2O_2/s induces increased gene expression and activity of the antioxidant defenses and increases PGC-1α expression, although the activation of the antioxidant defenses can not avoid the apparition of oxidative damage.

In conclusion, both the activation of the antioxidant defenses and the induction of oxidative damage induced by reactive oxygen species are closely related processes. The lower levels of reactive oxygen species that enhance the activity and expression of the antioxidant defenses also induce the apparition of oxidative damage in the cellular components. The intake of antioxidants in moderate dosages above the recommended dietary allowances is effective in enhancing the endogenous antioxidant defenses and decreasing the oxidative damage markers. Therefore, these nutrients are appropriate to increase the antioxidant protection and prevent the apparition of oxidative damage.

Lista de artículos originales / *List of original papers*

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II. Sureda A, Ferrer MD, Batle JM, Tauler P, Tur JA, Pons A. *Scuba diving increases erythrocyte and plasma antioxidant defenses and spares NO without oxidative damage*. Medicine & Science in Sports & Exercise (In Press).

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XI. Ferrer MD, Tauler P, Sureda A, Palacín C, Tur JA, Pons A. *Impaired mitochondrial antioxidant defences in variegate porphyria are accompanied with more inducible ROS production and oxidative damage.*

XII. Ferrer MD, Tauler P, Sureda A, Palacín C, Tur JA, Pons A. *A diet supplementation with vitamins E and C increases the lymphocyte antioxidant protection in women affected by variegate porphyria which presented impaired antioxidant defences*.

XIII. Ferrer MD, Tauler P, Sureda A, Palacín C, Tur JA, Pons A *Variegate porphyria induces plasma and neutrophil oxidative stress. Effects of a diet supplementation with vitamins E and C.*

XIV. Mestre A, Ferrer MD, Sureda A, Tauler P, Martínez E, Bibiloni MM, Micol V, Tur JA, Pons A. *Phytoestrogens enhance antioxidant enzyme defenses and reduce testosterone and estradiol circulating levels*.

XV. Ferrer MD, Sureda A, Mestre A, Tur JA, Pons A. *The double edge of reactive oxygen species as damaging and signaling molecules in HL60 cell culture.*

I. INTRODUCCIÓN

1. Estrés oxidativo y los elementos que participan

1.1. Especies reactivas

1.1.1. Especies reactivas de oxígeno

El oxígeno molecular, esencial para la vida, puede ser convertido en moléculas altamente reactivas, denominadas especies reactivas de oxígeno (ROS), entre las que destacan el peróxido de hidrógeno (H_2O_2), el anión superóxido (O_2^-) o el radical hidroxilo (OH^-). Todas estas especies reactivas se generan en una gran variedad de procesos celulares y están implicadas en una multitud de procesos fisiológicos tales como la función inmune y el envejecimiento, así como en el comienzo y la progresión de procesos patológicos. Entre las especies reactivas de oxígeno encontramos radicales libres (átomos o moléculas que contienen uno o más electrones desapareados) y especies no radicales pero igualmente reactivas. Los radicales libres se generan como productos de reacciones hemolíticas, heterolíticas o redox. Paralelamente a las ROS nos encontramos con las especies reactivas de nitrógeno (RNS), que también incluyen radicales libres así como especies reactivas no radicales.

El anión superóxido ($O_2^{\cdot-}$) se forma cuando el oxígeno molecular (O_2) capta un electrón, y se produce como intermediario en un gran número de reacciones bioquímicas [1]. Al tener una vida media relativamente larga puede difundir por la célula, de forma que se incrementa así el número de posibles dianas de reacción. A pesar de que es una especie impermeable a las membranas celulares, se ha descrito la posibilidad de que su protonación a pH fisiológico en las proximidades de las membranas, dando lugar a radical hidroperoxilo ($HO_2^{\cdot}$), facilitaría su tránsito a través de membranas [2]. Las principales fuentes intracelulares de $O_2^{\cdot-}$ son la cadena respiratoria, reacciones catalizadas por ciertas oxidasas como la xantina oxidasa (enzima que cataliza la oxidación de la xantina e hipoxantina a ácido úrico en el metabolismo de las purinas) [3] y la activación de células del sistema inmunitario como los neutrófilos y los macrófagos. Estas células del sistema inmune expresan el enzima NADPH oxidasa, que cataliza la generación de anión superóxido a partir de oxígeno y NADPH [4, 5].

El peróxido de hidrógeno (H_2O_2) se forma por dismutación del anión $O_2^{\cdot-}$, ya sea ésta espontánea o catalizada por el enzima superóxido dismutasa (SOD).

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H+ \rightarrow H_2O_2 + O_2$$

A pesar de ser un agente citotóxico, el H_2O_2 no posee un potencial oxidante muy elevado, y así no es capaz de oxidar el DNA o lípidos directamente, pero sí puede

inactivar ciertos enzimas [6]. A parte de por dismutación del $O_2^{\cdot-}$, el peróxido de hidrógeno también se puede generar durante reacciones enzimáticas como las catalizadas por los enzimas urato oxidasa y aminoácido oxidasas [7]. El H_2O_2 es un compuesto reactivo que puede generar otros radicales libres como el radical hidroxilo a través de reacciones catalizadas por metales como pueda ser la reacción de Fenton:

$$O_2^{\cdot-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

El radical hidroxilo ($OH^{\cdot}$) es una especie altamente reactiva con un fuerte potencial oxidante, y por tanto sólo es capaz de reaccionar con moléculas que se encuentren cercanas a su lugar de formación. Los radicales $OH^{\cdot}$ son considerados como las ROS más dañinas, y su reactividad es tal que resulta virtualmente imposible confirmar su existencia en organismos vivos más allá de detectar la presencia de productos específicos de su reacción [8].

Otra forma reactiva del oxígeno es el oxígeno singlete ($^1O_2$), una especie no radical que a nivel celular se puede formar a partir de la dismutación del anión superóxido en medio acuoso. Al igual que el radical $OH^{\cdot}$, el $^1O_2$ tiene una vida media muy corta, pero a diferencia del anterior es capaz de difundir a través de membranas.

1.1.2. Especies reactivas de nitrógeno

El radical óxido nítrico ($NO^{\cdot}$) es la especie reactiva de nitrógeno más abundante en los sistemas biológicos. Es un radical libre gaseoso sintetizado a partir de L-arginina por una familia de isoenzimas llamados óxido nítrico sintasas (NOS). De estos enzimas se han descrito hasta el momento tres isoformas, dos de ellas constitutivas (eNOS y nNOS, endotelial y neuronal, respectivamente) y una inducible (iNOS). La iNOS se encuentra presente en muchas células del sistema inmune, que una vez activadas producen grandes cantidades de NO, actuando como molécula de defensa contra agentes infecciosos [9]. Junto con este efecto antimicrobiano, el óxido nítrico participa en un elevado número de procesos fisiológicos tales como la regulación del flujo sanguíneo y la inhibición de la agregación plaquetaria, posee actividad antiinflamatoria y actúa como neurotransmisor [10-12].

El óxido nítrico puede reaccionar rápidamente con el radical $O_2^{\cdot-}$ dando lugar a peroxinitrito ($ONOO^-$):

$$O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^{-}$$

Esta reacción ocurre tres veces más rápido que la dismutación de superóxido a peróxido de hidrógeno, por lo que es la reacción predominante mientras coexistan

ambas especies reactivas. El peroxinitrito es un fuerte agente oxidante con la capacidad de dañar el DNA y producir la nitración de proteínas.

1.1.3. Otras especies reactivas

El hipoclorito (HOCl) es una especie reactiva formada por la acción enzimática de la mieloperoxidasa, un enzima principalmente presente en los neutrófilos que cataliza la formación de HOCl a partir de H_2O_2 y Cl^-.

$$H_2O_2 + Cl^- \rightarrow HOCl + OH^-$$

El HOCl puede dañar diferentes biomoléculas a partir de la oxidación de grupos tiol, lípidos, etc. En su forma ácida puede atravesar membranas y provocar la rotura y agregación de proteínas.

Los radicales peroxilo orgánicos ($ROO^{\cdot}$) se forman por la adición de una molécula de oxígeno a cualquier radical de carbono. El ataque de un radical a un compuesto hidrocarbonado supone la pérdida de un átomo de hidrógeno formando un radical centrado en el carbono altamente inestable que reacciona rápidamente con el oxígeno formando el radical peroxilo. A partir de hidroperóxidos orgánicos también se generan radicales alcoxilo ($RO^{\cdot}$).

1.2. Defensas antioxidantes

Para evitar los efectos deletéreos que causaría un exceso de especies reactivas, nuestro organismo contiene sistemas antioxidantes. Estos sistemas pueden actuar a dos niveles, ya sea reduciendo la producción de ROS o aumentando su detoxificación (o la de los productos de su reacción) una vez producidos. Así, pueden actuar controlando las concentraciones locales de oxígeno libre, quelando iones metálicos o inactivando otras sustancias prooxidantes, eliminando las especies activadas de oxígeno o transformándolas en moléculas menos activas, previniendo la formación de radicales o favoreciendo la transformación de las formas más reactivas en formas menos peligrosas, reparando el daño oxidativo e incrementando la eliminación de moléculas dañadas. Los antioxidantes se encuentran tanto en el medio extracelular como en el interior de la célula, y dentro de la célula se encuentran compartimentados en los diferentes orgánulos y espacios celulares. Entre estas defensas encontramos antioxidantes de bajo peso molecular, como el glutatión (GSH) o las vitaminas C y E, y los enzimas antioxidantes: catalasa, glutatión peroxidasa y superóxido dismutasa.

1.2.1. Defensas antioxidantes enzimáticas

Las células poseen una serie de enzimas cuya función es la transformación de las ROS en especies no reactivas y la reparación de los daños producidos por oxidación. Los principales enzimas antioxidantes son la catalasa, la glutatión peroxidasa y la superóxido dismutasa, pero también existen otros enzimas que complementan la función antioxidante como son la glutatión reductasa, peroxiredoxina o tioredoxina reductasa.

La superóxido dismutasa (SOD) es la primera línea de defensa frente a los radicales superóxido, ya que se encarga de la dismutación de estos radicales a peróxido de hidrógeno (mucho menos reactivo que el $O_2^{\cdot-}$) y oxígeno. En mamíferos se han descrito tres isoformas, todas ellas con un metal de transición en su centro activo que permite la rotura catalítica del $O_2^{\cdot-}$ [13]. La isoforma SOD1 (o Cu/Zn-SOD) requiere cobre-zinc como cofactor y se encuentra localizada en el citoplasma y el espacio intermembrana de la mitocondria. La isoforma SOD2 (o Mn-SOD) requiere manganeso como cofactor y se encuentra localizada en la matriz mitocondrial. Finalmente, la SOD3 (o ecSOD) también requiere cobre-zinc como cofactor, pero se encuentra localizada en el espacio extracelular.

La glutatión peroxidasa (GPx) es un enzima tetramérico que posee un residuo de seleno-cisteína en cada una de sus subunidades y cataliza la reducción de H_2O_2 e hidroperóxidos (ROOH) a H_2O y alcohol (ROH), respectivamente, usando glutatión reducido (GSH) como donador electrónico, el cual es oxidado a disulfuro de glutatión (GSSG), también llamado glutatión oxidado [6, 14].

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O$$

Hasta el momento se han descrito cinco isoformas de glutatión peroxidasa (GPx1-GPx5) [15] que catalizan la misma reacción pero difieren en la especificidad de sustrato (diferentes hidroperóxidos) y en la localización celular (citosólica, mitocondrial, etc.) [16]. Esta variedad en la especificidad de sustrato y en la localización optimiza la función de la glutatión peroxidasa como defensa antioxidante, que puede reducir tanto el H_2O_2 como hidroperóxidos orgánicos complejos resultado de la oxidación de lípidos de membrana, proteínas y ácidos nucleicos [17]. Puesto que la disponibilidad de GSH debe ser continua para la óptima función de la GPx, la célula debe disponer de un mecanismo de regeneración de GSH a partir de GSSG. Para ello cuenta con la glutatión reductasa (GRd), un flavoenzima que se encarga de la reducción de GSSG a GSH utilizando NADPH como cofactor [18].

La catalasa cataliza, al igual que la GPx, la descomposición del H_2O_2 a H_2O, pero en su caso no utiliza ningún otro sustrato.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

La catalasa es un homotetrámero ampliamente distribuido por toda la célula [14, 19]. Para funcionar necesita la presencia de hierro como cofactor, que se encuentra unido al centro activo del enzima formando parte de un grupo hemo. A pesar de compartir sustrato con la GPx, la catalasa posee una menor afinidad por el H_2O_2 a bajas concentraciones (GPx K_m = 1 μM vs catalasa K_m = 1 mM) [20].

Junto con estos enzimas antioxidantes principales, la célula contiene otros enzimas que pueden contribuir, ya se de forma directa o indirecta, al mantenimiento del balance redox. El sistema de la tioredoxina está compuesto por tioredoxina (TRX) y tioredoxina reductasa. La TRX es una proteína de 12-kDa altamente conservada que se encuentra en el citoplasma (TRX1) y en la mitocondria (TRX2) [21]. Su función principal es la de mantener las proteínas en su estado reducido a través de su actividad disulfuro reductasa, pero también se le atribuyen otras funciones tales como reducción de factores de transcripción y control de la apoptosis. Una vez oxidada, la TRX es reciclada a su forma reducida por la actividad de la TRX reductasa, que utiliza los electrones provenientes de la oxidación del NADPH. La TRX reductasa también actúa como un enzima antioxidante a través de la reducción directa de hidroperóxidos y del reciclaje de la vitamina C [22].

La glutaredoxina (GRX) es una tiodisulfuro oxidoreductasa implicada en la protección y reparación de grupos tiol tanto proteicos como no proteicos en situaciones de estrés oxidativo [21]. Su mecanismo de acción consiste en transferir electrones del NADPH a sustratos disulfuro en un ciclo catalítico acoplado a glutatión y glutatión reductasa.

1.2.2. Otras proteínas antioxidantes

La mitocondria es una fuente importante de especies reactivas de oxígeno, principalmente a través de la cadena respiratoria. Para mantener controlada esta producción continua de ROS, la mitocondria cuenta con toda una serie de proteínas con características antioxidantes. Entre estas podemos destacar las proteínas desacoplantes (UCP), presentes en la membrana interna de las mitocondrias. La UCP-1 se encuentra únicamente en adipocitos marrones y participa en la termogénesis. Las UCP-2 (sistema linfoide, macrófagos, islotes pancreáticos), UCP-3 (presente principalmente en el músculo esquelético), UCP-4 y UCP-5 (presentes en el cerebro) a pesar de tener actividad desacoplante, no participan en la termogénesis. Se ha

comprobado que tanto la UCP-2 como la UCP-3 disminuyen la producción de ROS en la mitocondria [23]. UCP-3 también ha sido detectada en bazo y timo, tanto en humanos como en ratas. En ratas se ha detectado también en linfocitos, donde aumenta la concentración de la proteína tras el ayuno [24].

En humanos la UCP-3 presenta dos tránscritos, UCP-3_L y UCP-3_S, que codifican para la UCP-3 larga (312 aminoácidos, 34 kDa) y la UCP-3 corta (275 aminoácidos, 30 kDa), y que difieren únicamente en la presencia o ausencia de 37 residuos C-terminales (parte del sexto y último dominio transmembrana). De estas dos isoformas parece ser que UCP-3_S tiene una mayor actividad intrínseca [25, 26]. Se han encontrado correlaciones positivas entre la expresión de UCP-3 y el gasto de energía, aunque parece que la función primaria de ésta no es la regulación del metabolismo energético, ya que por ejemplo también el ayuno activa su expresión. En sujetos inactivos físicamente se ha encontrado un incremento en la expresión de UCP-3. Sujetos entrenados presentan una menor expresión de UCP-3, y esta menor expresión se correlaciona con una mayor eficiencia energética. Por el contrario, el ejercicio agudo incrementa la expresión de UCP-3 en músculo esquelético, y esta mayor expresión parece estar más relacionada con los cambios en el metabolismo de los ácidos grasos que con el gasto de energía: tras consumirse el glucógeno se liberan ácidos grasos en una cantidad mayor a la que se puede oxidar, y el exceso de ácidos grasos en la matriz sería recogido por la UCP-3 y transportado al exterior de la mitocondria [27]. El hecho de tener una menor cantidad de glucógeno en el músculo provoca que el aumento en la expresión de UCP-3 tras el ejercicio sea mayor que cuando el músculo tiene una disponibilidad normal de glucógeno [28]. En este sentido, también se ha comprobado que una dieta pobre en carbohidratos incrementa la expresión de UCP-3 respecto a una dieta rica en carbohidratos [29].

En el músculo esquelético UCP-3 presenta unos niveles mayores de expresión en las fibras musculares glucolíticas (que tienen una menor capacidad para oxidar ácidos grasos) que en las oxidativas, y los incrementos en la expresión en respuesta al ayuno o a una dieta rica en grasas también son más pronunciados. En situaciones en las que aumenten los niveles de ácidos grasos libres, éstos difundirán hacia el interior de la mitocondria, donde se desprotonarán debido al gradiente protónico, y una vez en forma de anión no pueden difundir al exterior. UCP-3 puede transportar los aniones de ácidos grasos hacia el exterior, y así se postula una de las funciones de la proteína sería la de proteger a la mitocondria frente a la acumulación de ácidos grasos [30].

El transporte de protones por parte de UCP-2 y UCP-3 se realiza únicamente en presencia de activadores. Entre estos activadores se encuentran la ubiquinona, el superóxido, alquenales (productos de la peroxidación lipídica), retinoides, etc. Los

alquenales parecen ser los activadores directos, mientras que el resto induce la producción de alquenales. Por el contrario, los nucleótidos de purina, como el ATP o el GDP, inhiben la actividad de UCP-2 y UCP-3 [31].

El anión superóxido, tanto el generado fuera de la mitocondria como el generado en la matriz, podría ser uno de los principales activadores de las UCP. El desacoplamiento de la cadena respiratoria reduce tanto el potencial de membrana como el gradiente de pH, y esto a su vez reduce la producción de anión superóxido por parte del complejo I. Así, en condiciones de alta producción de superóxido se activan las UCP y aumenta el desacoplamiento, lo que provoca una disminución en la producción de superóxido [32]. Sin embargo los resultados obtenidos hasta el momento no son del todo concluyentes y a día de hoy todavía se discuten los mecanismos de regulación de las proteínas desacoplantes [33].

Otras proteínas cuya presencia se ha descrito recientemente en la mitocondria y que parecen tener una función antioxidante son las sirtuinas. Las sirtuinas son histona desacetilasas de clase III que difieren del resto de histona desacetilasas por su dependencia del NAD. En humanos se han descrito siete sirtuinas (SIRT1-7) [34]. SIRT1 se encuentra en el núcleo, y es la sirtuina que guarda más semejanzas con la Sir2 de levadura. SIRT1 une y desacetila p53, NF-κB e histonas. En mamíferos es activada por restricción calórica en varios tejidos [35]. SIRT2 se localiza en el citoplasma y desacetila α-tubulinas. Su concentración se incrementa en la mitosis, lo que sugiere un papel en la regulación del ciclo celular. SIRT 3, SIRT4 y SIRT5 se localizan en la mitocondria. SIRT6 y SIRT7 se localizan en el núcleo, pero en regiones diferentes a SIRT1. SIRT6 se encuentra asociada a heterocromatina, mientras que SIRT7 se encuentra localizada en el nucleolo. Estas dos últimas sirtuinas no desacetilan p53 ni histonas, pero se han encontrado asociadas a los cromosomas durante la mitosis. La sirtuina Sir2 de levaduras parece estar relacionada con el período de vida. En cambio, la sobreexpresión de las siete sirtuinas no ha mostrado tener ningún efecto sobre el período de vida de las células humanas. Aun así la expresión de SIRT1 endógena disminuye progresivamente a medida que las células se van dividiendo en cultivo [36].

SIRT3 es sintetizada en el citoplasma en forma de precursor inactivo y es transportada a la matriz mitocondrial (25 aminoácidos N-terminal), donde se procesa proteolíticamente para activar su actividad enzimática [37, 38]. Ya que en la mitocondria no hay histonas, se supone que SIRT3 desacetila otras dianas. En contraste con el citoplasma, donde la concentración de NAD puede variar en respuesta a la disponibilidad de ATP, la concentración de NAD en la matriz

mitocondrial permanece estable. Estímulos apoptóticos, ROS o un incremento de los niveles de Ca^{2+} podrían inhibir la función de SIRT3 por varios mecanismos: depleción de NAD, aumento de su hidrólisis, formación de nicotinamida e inhibición del transporte de SIRT3 a la matriz mitocondrial [37]. Se ha detectado RNA de SIRT3 en músculo, hígado , cerebro, pulmón, riñón y páncreas [38].

SIRT3 tiene una mayor expresión en el tejido adiposo marrón (TAM) que en el blanco (TAB). Su expresión aumenta tras la restricción calórica o la exposición al frío. La sobreexpresión de SIRT3 provoca un incremento en la expresión de genes como UCP1, PGC-1α, subunidades II y IV de la citocromo oxidasa y ATP sintetasa. La activación en la expresión de UCP1 parece que está mediada a través del incremento de expresión de PGC-1α, activado a su vez vía CREB. Se ha observado que la sobreexpresión de SIRT3 induce un incremento en la respiración y el desacoplamiento, y a la vez reduce la formación de ROS [39]. Los mecanismos a través de los cuales la SIRT3 incrementa la respiración no están todavía claros, pero los últimos resultados apuntan a la desacetilación y activación de enzimas mitocondriales implicados en el metabolismo respiratorio y el control del ciclo del ácido cítrico, tales como la isocitrato deshidrogenasa [40] (enzima que produce NADPH, importante para la regeneración de antioxidantes que dependen de NADPH), glutamato deshidrogenada [40] y acetil coenzima A sintetasa 2 [41]. Es evidente, por tanto, que la SIRT3 puede jugar un papel importante en los mecanismos mitocondriales de producción y eliminación de especies reactivas.

La función de SIRT3 todavía permanece desconocida, pero se han encontrado relaciones entre determinados genotipos de SIRT3 (cuya localización cromosómica se encuentra próxima a la de 4 genes implicados en el envejecimiento) y la longevidad en hombres pero no en mujeres. Uno de los polimorfismos se encuentra en la posición 477 (exón 3), donde se ha encontrado una transversión silenciosa (G/T). Los portadores del genotipo TT tienen una mayor función de supervivencia que los portadores del GG o GT [42]. Los mismos autores encontraron posteriormente un polimorfismo en una VNTR (72 pb) del exón 5, que actúa como *enhancer*, y cuya actividad varía en función del número de repeticiones y la secuencia. La distribución de los diferentes alelos varía en función de la edad de la población estudiada, lo que podría indicar una relación entre el genotipo de SIRT3 y la longevidad [43].

1.2.3. Defensas antioxidantes no enzimáticas

Las defensas antioxidantes no enzimáticas comprenden un amplio y heterogéneo conjunto de moléculas orgánicas que ayudan al mantenimiento del equilibrio redox celular o extracelular mediante su acción detoxificadora de especies reactivas o sus productos. Estas defensas se pueden clasificar en antioxidantes endógenos (glutatión, ubiquinona, bilirrubina, etc.), sintetizados por las células de nuestro organismo, y antioxidantes dietéticos (vitaminas E y C, carotenoides, compuestos fenólicos, etc.), que al no ser capaces nuestras células de sintetizarlos deben ser incluidos en la dieta.

La vitamina E (mayoritariamente en la forma α-tocoferol, aunque existen al menos ocho isómeros estructurales del tocoferol) [44] es un antioxidante liposoluble localizado en membranas y entornos lipofílicos, lo que lo convierte en especialmente eficiente a la hora de desactivar radicales libres originados en la membrana mitocondrial interna y otras membranas. Su función es la de reaccionar con radicales peroxilo deteniendo así las reacciones de autooxidación, y tras esta reacción se forma un radical de tocoferol que debe ser reciclado por otros antioxidantes tales como la vitamina C o el glutatión [45-47]. Recientemente también se le ha atribuido una importante función como regulador de la expresión génica [48] y de las vías celulares de transducción de señal [49]. Los genes regulados por vitamina E se pueden clasificar en cinco grupos: genes que participan en la captación y degradación de tocoferoles, genes asociados a la captación de lípidos y al desarrollo de la aterosclerosis, genes que modulan la expresión de proteínas extracelulares, genes relacionados con la inflamación y adhesión celular, y genes que codifican proteínas que participan en la señalización celular y la regulación del ciclo celular [48]. Sin embargo, resulta llamativo que hasta el momento no se ha detectado ninguna relación directa entre la vitamina E y la expresión de genes antioxidante [50].

La vitamina C (ácido ascórbico) es un antioxidante hidrosoluble que se encuentra principalmente en el citoplasma y en los fluidos extracelulares. Debido a que su pKa es 4,25, a pH fisiológico se encuentra en la forma desprotonada o ascorbato. Sus propiedades químicas le permiten reaccionar directamente con el $O_2^{\cdot-}$ y el $OH^{\cdot}$ en la fase acuosa del plasma, previniendo así la aparición de daño oxidativo en las membranas de las células sanguíneas [51, 52]. La vitamina C también se encarga de reducir los radicales de tocoferol a su forma activa, oxidándose a su vez a la forma dehidroascorbato, que puede ser reducida de nuevo por glutatión o por el ciclo redox del ácido dihidrolipoico [6].

Así mismo, también se han descrito numerosas moléculas con actividad antioxidante que son ingeridas con los alimentos y que, si bien no son consideradas como vitaminas, son objeto de investigación debido a su potente efecto antioxidante. Entre ellas podemos incluir los carotenoides, como el β-caroteno, que actúan a nivel de membranas y lipoproteínas eliminando radicales superóxido y peroxilo y oxígeno singlete [53]. Las propiedades antioxidantes de los carotenoides derivan de su distribución estructural, que consiste en largas cadenas de dobles enlaces conjugados, lo que permite la neutralización de los diferentes tipos de ROS. Debido tanto a su localización celular en membranas como a su potencial detoxificador, los carotenoides son considerados como unos eficientes antioxidantes contra la peroxidación lipídica [54]. Otras sustancias antioxidantes ingeridas con los alimentos son los compuestos fenólicos como la antocianidina, las isoflavonas o los taninos, que pueden capturar radicales libres o disminuir su producción, así como actuar como quelantes de metales [55]; o el ácido lipoico, capaz de reaccionar con especies reactivas de oxígeno así como con otros antioxidantes como las vitaminas E y C o el glutatión, regenerándolos a partir de sus formas oxidadas [56]. Los fitoestrógenos son compuestos similares a los estrógenos humanos pero presentes en los vegetales. Estos compuestos, que se encuentra distribuidos ampliamente en el reino vegetal, poseen propiedades antioxidantes [57-61] pero también exhiben efectos antiproliferativos, citotóxicos, antimetastáticos e inmunomoduladores [62-65]. La similitud estructural con los estrógenos humanos permite que los fitoestrógenos también actúen como agonistas o antagonistas de las hormonas estrogénicas, entre ellas el 17-β-estradiol, a través de la interacción con los receptores de estrógenos alfa (ERα) y beta (ERβ) [66, 67].

De los antioxidantes no enzimáticos endógenos, el glutatión es el más abundante en las células. El glutatión (γ-glutamilcisteinilglicina) es la fuente de tiol no proteico más abundante en las células, presente a concentraciones intracelulares en el rango de milimolar, y cuyas principal función es proteger a los tejidos del daño oxidativo manteniendo el medio intracelular en un estado reducido [18]. El glutatión reducido (GSH) participa en la reducción de peróxidos por la glutatión peroxidasa, la neutralización de $OH^{\cdot}$ y $^1O_2$ y en la reducción de semihidroascorbato y radicales tocoferol [17]. En todos estos procesos el GSH se transforma en glutatión oxidado (GSSG), y éste debe ser rápidamente reciclado a GSH puesto que la acumulación de GSSG es tóxica ya que puede provocar la unión de proteínas y DNA por puentes disulfuro [18]. El cociente GSSG/GSH se utiliza habitualmente como un indicador del estado redox celular.

El coenzima Q_{10} (ubiquinona) es también considerado como una molécula antioxidante. Se sintetiza en todas las células y es un componente esencial de la cadena de transporte electrónico mitocondrial, aunque también se encuentra en las membranas celulares [6]. Se ha comprobado que el coenzima Q_{10} puede actuar in vitro como un antioxidante no enzimático neutralizando radicales $RO_2^{\cdot\cdot}$, inhibiendo la peroxidación lipídica, reciclando el a-tocoferol y el ascorbato [68, 69], y previniendo los efectos prooxidantes del a-tocoferol [70]. Sin embargo su contribución a la defensa antioxidante en condiciones fisiológicas aún no está clara.

Algunos productos finales del metabolismo como el ácido úrico, que deriva del metabolismo de las purinas, o la bilirrubina, que deriva del catabolismo del grupo hemo, también tienen propiedades antioxidantes. El ácido úrico es un potente neutralizador de radicales peroxilo e hidroxilo y de oxígeno singlete, a la vez que es capaz de quelar iones metálicos como el hierro y el cobre y así previene la aparición de radicales hidroxilo por la reacción de Fenton [71]. La bilirrubina, por su parte, posee un fuerte potencial antioxidante contra radicales peroxilo y peróxido de hidrógeno. Este potencial parece resultado de un ciclo de amplificación por el cual, una vez ha actuado como antioxidante la bilirrubina es oxidada a biliverdina, que de nuevo es reciclada a bilirrubina vía biliverdina reductasa [72].

Finalmente, otros sistemas antioxidantes consisten en proteínas que transportan y secuestran posibles agentes prooxidantes como el hierro (transferrina, ferritina), el cobre (ceruloplasmina) o el grupo hemo (haptoglobina). El reciclaje de proteínas, la degradación de proteínas modificadas y la síntesis de nuevas proteínas funcionales, además de los procesos de reparación del DNA también pueden considerarse como parte del sistema de defensa antioxidante [73].

1.3. Equilibrio redox y estrés oxidativo

En condiciones de equilibrio, la tasa de generación de especies reactivas se encuentra neutralizada por una similar tasa de eliminación de dichas especies por los mecanismos antioxidantes anteriormente explicados. Cuando por el contrario las especies reactivas se producen a una velocidad mayor a la de su eliminación, éstas pueden dañar las estructuras celulares como consecuencia de la oxidación de los diferentes tipos de macromoléculas. Esta situación de desequilibrio en la homeóstasis de las especies reactivas se conoce con el nombre de estrés oxidativo. Así, una situación de estrés oxidativo se caracteriza por un incremento en la formación de radicales y otras especies oxidantes, un descenso de antioxidantes de bajo peso molecular, un desequilibrio del balance redox celular, y la aparición de daño oxidativo

en componentes celulares. En condiciones de estrés oxidativo, todas las biomoléculas celulares son susceptibles de sufrir modificaciones oxidativas.

Los ácidos grasos poliinsaturados presentes en las membranas celulares son especialmente susceptibles de ser atacados por los radicales libres, especialmente por el radical $OH^{\cdot}$, a través de reacciones de peroxidación lipídica. Estas reacciones de iniciación consisten en la extracción de un electrón al ácido graso, lo que conduce a la formación de un radical peroxilo ($LOO\cdot$). Cuando el ácido graso bajo ataque de los radicales se convierte en un radical lipídico con dobles enlaces conjugados, éstos enlaces resultan débiles y pueden reaccionar fácilmente con el oxígeno produciendo radicales peroxilo y finalmente peróxidos lipídicos. Estos peróxidos se suelen descomponer rápidamente hasta formar aldehídos (4-hidroxinonenal y malondialdehído), que pueden unirse a proteínas, otros lípidos, carbohidratos y DNA, alterando su estructura y su función [6]. La propia función del lípido también queda comprometida tras su peroxidación. Los lípidos de membrana, esenciales en el mantenimiento de la estructura y el transporte transmembrana de determinadas sustancias, adquieren un mayor carácter hidrofílico tras la peroxidación. Este cambio en su comportamiento químico altera la habilidad para transferir selectivamente metabolitos a través de la membrana. Como resultado puede entrar al interior de la célula un exceso de agua que conduce a un proceso de inflamación celular. A su vez esta inflamación puede inducir una mayor producción de radicales superóxido, contribuyendo así a una alimentación positiva que propaga las reacciones de peroxidación [74]. Debido a que los radicales lipídicos intermediarios que se forman durante la propagación son muy inestables y reaccionan rápidamente con otros compuestos, la peroxidación lipídica se suele determinar a través de los productos finales de reacción tales como el malondialdehído (MDA), el 4-hidroxinonenal (4-HNE) o las sustancias reactivas del ácido tiobarbitúrico (TBARS).

Las proteínas también son susceptibles de reaccionar con las especies reactivas de oxígeno, debido a su compleja estructura y al elevado número de grupos funcionales oxidables. Las especies reactivas que reaccionan principalmente con las proteínas son el H_2O_2, el radical $OH^{\cdot}$ y el $NO^{\cdot}$. La modificación oxidativa de las proteínas puede ocurrir por dos mecanismos diferentes. En primer lugar se puede producir una oxidación catalizada por metales, lo que conlleva la formación de grupos grupos carbonilo en las cadenas laterales [75, 76]. Los grupos carbonilo son grupos aldehídos y cetonas introducidos en la proteína por la mencionada oxidación catalizada por metales, pero también a través de la reacción con carbohidratos y lípidos [77]. Prolina, arginina, lisina e histidina son aminoácidos que pueden sufrir este tipo de oxidación. Por otro lado aminoácidos como la metionina, histidina, triptófano y

cisteína pueden ser atacados por radicales peroxilo y alcoxilo en ausencia de metales. En este proceso se puede llegar a producir la degradación oxidativa de la proteína por rotura del enlace peptídico. La oxidación de las proteínas conduce a una modificación de la estructura de éstas, lo que en última instancia puede acabar modificando su función, llevando incluso a la pérdida de la actividad enzimática en el caso de que las proteínas dañadas sean enzimas.

Las especies reactivas también son capaces de reaccionar con el DNA. El proceso de oxidación de DNA tiene lugar continuamente en la célula sin que sea necesaria la presencia de una situación de estrés oxidativo. El DNA oxidado es relativamente abundante en las células humanas, y parece ser que estas lesiones oxidativas en las bases del DNA se van acumulando con la edad [78, 79]. El ataque de las especies reactivas puede producir diferentes tipos de daño oxidativo en el DNA, entre los que destacan la modificación de las bases o los nucleótidos, la aparición de roturas en una o las dos cadenas y la aparición de entrecruzamientos DNA-proteína. El producto más común de la oxidación de las bases de DNA es la 8-hidroxi-7,8-dihidro-2'-deoxiguanosina (8-OHdG), una lesión que la célula es capaz de retirar rápidamente del DNA y del acervo de nucleótidos por diferentes mecanismos. La oxidación del DNA mitocondrial como consecuencia de un incremento en la producción mitocondrial de ROS parece implicada en el proceso de envejecimiento normal del ser humano, pero también en el desarrollo de diferentes patologías tales como el cáncer, la enfermedad de Parkinson, esclerosis lateral amiotrófica, enfermedad de Alzheimer y enfermedad de Huntintgton [80, 81], así como también puede estar implicada en la inducción de fallos cardíacos asociados a la edad [82]. La inducción de daño en el DNA se mide por diferentes técnicas. Las roturas de la cadena de DNA se suelen determinar por el método del comet assay, una técnica de electroforesis en microgel que permite la detección de daño en el DNA en células individuales con una elevada sensibilidad [83]. La oxidación de las bases se mide a partir de la determinación de los niveles de 8-OHdG (especialmente en orina) por HPLC [84, 85].

Después de un período de producción excesiva de ROS, las células y tejidos poseen mecanismos para reestablecer el estado redox original. La producción de NO, por ejemplo, está sujeta a regulación negativa de retroalimentación ya que el propio NO inhibe al enzima que lo sintetiza, la NOS. Otro mecanismo de homeostasis redox está basado en la inducción por ROS de cascadas de señalización que conducen a la expresión de enzimas antioxidantes o a incrementar el transporte de cisteína, lo que facilita un incremento en los niveles de glutatión. Mientras las tasas de producción y eliminación de ROS se mantengan constantes, las células y tejidos se encuentran en un estado estable. La señalización redox requiere que se desequilibre este balance, ya

sea incrementando las concentraciones de ROS o disminuyendo la actividad de los sistemas antioxidantes. Este evento prooxidante puede derivarse de la activación regulada de la producción endógena de especies reactivas o de la exposición a condiciones de estrés generadas por factores ambientales. Si el incremento en las ROS es relativamente pequeño, la respuesta antioxidante suele ser suficiente para compensar este incremento y para volver al equilibrio original. Así, las manifestaciones fisiológicas de regulación redox implican incrementos temporales de las condiciones oxidativas de la célula, pero a largo plazo los mecanismos tienden a mantener un estado estable denominado homeóstasis redox. Sin embargo, bajo ciertas condiciones la producción de ROS se ve incrementada de forma más prolongada, y la respuesta antioxidante puede no ser suficiente para devolver el sistema a las condiciones originales de homeóstasis. En estos casos, el sistema puede volver a alcanzar un equilibrio, pero el nuevo estado puede estar asociado a concentraciones de ROS más elevadas y a diferentes patrones de expresión génica. Para mantener esta homeóstasis redox es necesaria la presencia de mecanismos reguladores capaces de detectar los niveles de ROS y RNS.

1.4. Especies reactivas de oxígeno como mensajeros celulares

Durante los últimos años las especies reactivas han dejado de verse únicamente como las causantes de la oxidación de biomoléculas y la inducción de estrés oxidativo, ya que se ha comprobado que son un componente imprescindible en numerosas funciones fisiológicas. Entre estas funciones podemos destacar la regulación del tono vascular, regulación de las funciones controladas por la concentración de oxígeno, potenciación de la transducción de señal a partir de receptores de membrana y la regulación de la respuesta antioxidante contra el estrés oxidativo explicada en el apartado anterior. Las ROS pueden actuar a través de muchas vías de transducción de señal diferentes, interaccionando con elementos señalizadotes tales como calcio, tirosina quinasas (PTK), tirosina fosfatasas (PTP), serina/treonina quinasas y fosfolipasas. Las cascadas de fosforilación están implicadas en numerosos mecanismos de transmisión de señales extracelulares desde la membrana plasmática hasta el núcleo. Entre las principales vías de regulación en las que participan las ROS encontramos la activación de factores de transcripción (NF-kB, AP-1, p53), la activación de las vías de las MAPK y del PI_3K/Akt, etc. Si bien hay toda una serie de agentes químicos que pueden servir de moléculas señalizadotas en respuesta al estrés oxidativo, el más importante parecer ser el H_2O_2 por varios motivos: se produce constantemente en la cadena respiratoria, es relativamente

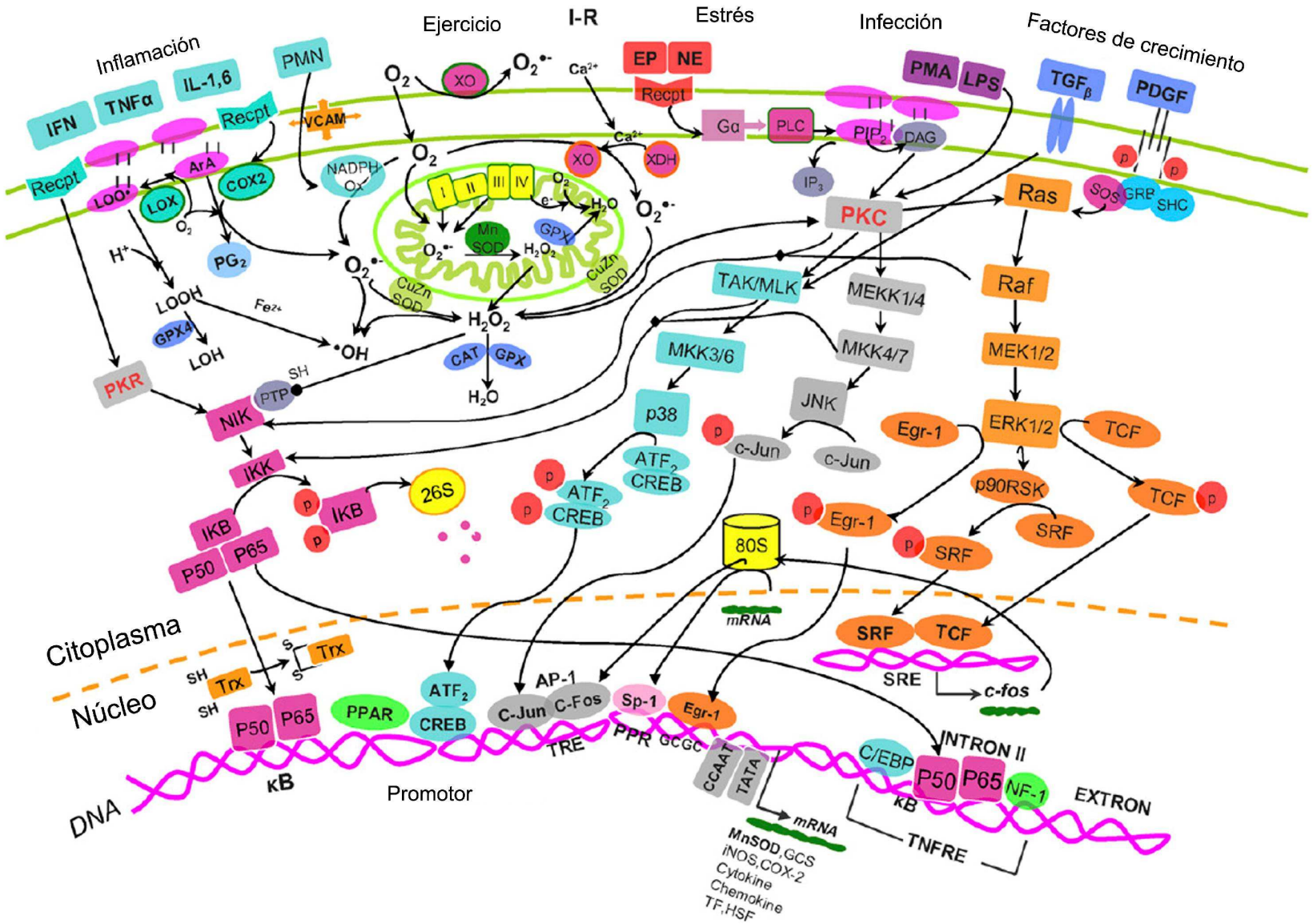

Figura 1. Esquema general de mecanismos de producción de ROS en respuesta a estímulos fisiológicos, sistemas de defensa antioxidante y vías de señalización redox. Extraída de Ji, 2007.

estable, es un oxidante capaz de oxidar diferentes grupos químicos (sulfidrilos, hidroxilos, sulfóxidos, etc.) a la vez que no es muy destructivo, y es una molécula capaz de atravesar algunas membranas biológicas, pero no todas [86].

1.4.1. Generación de especies reactivas de oxígeno como segundos mensajeros

Las ROS pueden surgir de fuentes tanto intracelulares como extracelulares. Las ROS extracelulares pueden iniciar la cascada de señalización a través de la activación de receptores de factores de crecimiento y citoquinas sin la necesidad de que los ligandos del receptor estén presentes [87, 88] o a través de la generación de peróxidos lipídicos en la membrana celular [89]. Por otro lado, toda una serie de estímulos pueden provocar la producción intracelular de ROS. La producción de ROS por NAD(P)H oxidasas juega un papel muy importante en la regulación de las cascadas de señalización celular en diferentes tipos de células no fagocíticas tales como fibroblastos, células endoteliales, células de la musculatura lisa vascular y miocitos cardíacos [90]. Las NAD(P)H oxidasas del sistema cardiovascular son enzimas asociados a membrana que parecen utilizar tanto NADH como NADPH [91]. Estos enzimas se ven inducidos por angiotensina, trombina, factor de crecimiento derivado de plaquetas (PDGF), factor de necrosis tumoral alfa (TNF-a) e interleuquina-1 (IL-1) [90, 91]. Otro enzima que puede contribuir a la producción intracelular de ROS con fines reguladores es la lipoxigenasa (dioxigenasa que oxida ácidos grasos poliinsaturados a hidroperoxilos con dobles enlaces conjugados y que está implicada en la biosíntesis de leucotrienos). Los metabolitos oxidados generados por la lipoxigenasa pueden cambiar el balance redox intracelular e inducir vías de transducción de señal y expresión génica.

1.4.2. Efectos de las especies reactivas de oxígeno en las vías de señalización celular

Una vez las ROS han sido producidas, sus efectos reguladores suelen comenzar con la activación de diferentes quinasas o por la inactivación de fosfatasas. Se ha comprobado que el H_2O_2 es capaz de incrementar la actividad de diferentes tirosina quinasas así como el nivel de fosforilación de varias proteínas celulares [90]. Las cascadas de señalización de las MAPK (proteínas quinasa activadas por mitógenos) están reguladas por la fosforilación y desfosforilación de residuos de serina y treonina, y responden a la activación por receptores con actividad tirosina quinasa, a receptores de citoquinas y factores de crecimiento y a receptores acoplados a

proteínas G. Las MAP quinasas que parecen más susceptibles a regulación por ROS y el estado tiol/disulfuro celular son JNK y p38 [90], y su activación por ROS está mediada, al menos en parte, por la activación oxidativa de tirosina quinasas de la familia Src [92].

Las proteínas quinasa C (PKC) son serina treonina quinasas implicadas en la transducción de señal de varias rutas que regulan la transcripción génica y el control del ciclo celular. Algunas isoformas de PKC, como la PKC-α, son activadas por diacilglicerol (DAG) y por agentes miméticos de éste como los ésteres de forbol (como el PMA). Se ha comprobado que diferentes isoformas de PKC pueden ser activadas por H_2O_2 en ausencia de DAG, en un proceso que implica la fosforilación de una tirosina en el dominio catalítico [93, 94]. Por otro lado, la oxidación del dominio carboxiterminal inactiva la PKC, lo que explicaría la regulación redox dual por la que bajos niveles de ROS activan su actividad mientras que niveles más altos la inhiben [95].

1.4.3. Efectos de las especies reactivas de oxígeno en la activación de factores de transcripción

Numerosos factores de transcripción exhiben sensibilidad redox. De éstos, los más estudiados han sido el AP-1 y el NF-kB. El factor de transcripción AP-1 está compuesto por homo- o heterodímeros de miembros de las familias Fos y Jun (normalmente es un heterodímero de c-Fos y c-Jun). Está implicado en procesos de diferenciación, crecimiento y estrés. En linfocitos T, AP-1 está implicado en la expresión de interleuquina-2 y de otros genes importantes en la función inmune. Muchos estímulos que inducen estrés oxidativo conllevan la activación de AP-1 por al menos dos mecanismos diferentes: regulación transcripcional de los genes que codifican para c-Fos y c-Jun y fosforilación de las proteínas c-Fos y c-Jun. La expresión de c-Fos y c-Jun se ve inducida por bajos niveles de H_2O_2, $O_2^{-}\cdot$ y NO [90]. La actividad como factor de transcripción de AP-1 y su capacidad de unión al DNA se ven potenciadas por fosforilación, llevada a cabo por la quinasa JNK y quinasas de la vía de ERK. JNK es una MAPK que fosforila residuos de serina en el dominio de transcativación de c-Jun [96, 97]. En situaciones basales, JNK se encuentra asociada a la glutatión-S-transferasa, lo que mantiene inhibida la actividad quinasa. Los agentes oxidantes promueven la disociación de este complejo, y por tanto potencian la activación de JNK [98].

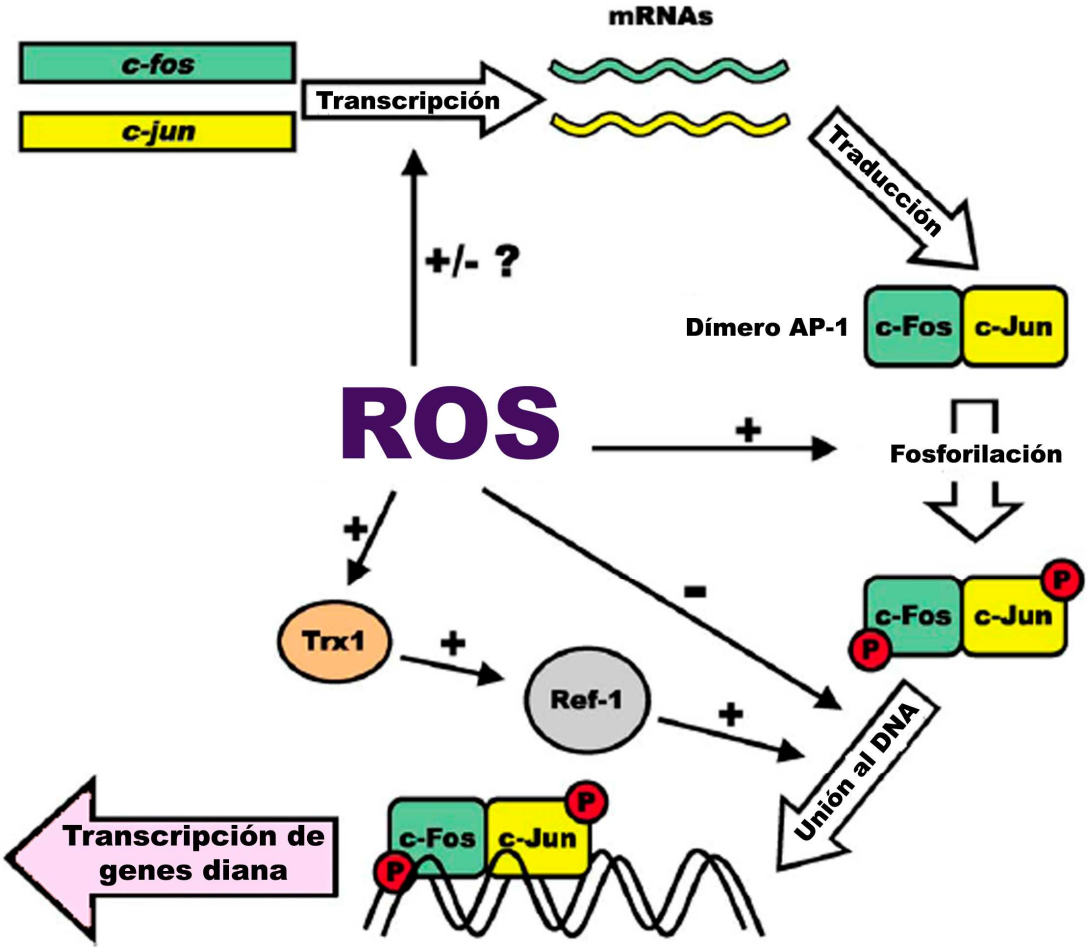

Figura 2. Mecanismos de regulación redox de la actividad del factor de transcripción AP-1. Extraída de Jackson *et al*, 2002.

El factor nuclear-kB (NF-kB) es un factor de transcripción que juega un papel fundamental en la expresión inducible de numerosos genes que codifican para proteínas implicadas en la defensa inmune y la inflamación (genes de fase aguda, receptores de membrana, citoquinas, etc.) [99] y más recientemente se ha comprobado que también activa la expresión de genes como la Mn-SOD, citocromo oxidasa e iNOS. El NF-kB es un heterodímero presente de forma latente en el citoplasma, donde se encuentra estabilizado por otra proteína inhibidora, la ikB [100]. Tras la estimulación de la célula por los estímulos adecuados IkB se disocia de NF-kB, permitiendo que el NF-kB libre sea translocado al núcleo, donde se unirá a los elementos reguladores de la región promotora en sus genes diana, provocando la activación del gen. Entre los estímulos que pueden conducir a la activación de NF-kB se encuentran citoquinas inflamatorias (TNF-a, IL-1), radiación, ésteres de forbol, infecciones virales o bacterianas y ciertos oxidantes [101]. Casi todos estos estímulos tienen la característica común de que provocan un incremento de los niveles

intracelulares de especies reactivas de oxígeno, especialmente $O_2^{\cdot-}$, H_2O_2 y $OH^{\cdot}$ [102]. Uno de los primeros acontecimientos en el proceso de activación del NF-kB es la fosforilación de IkB por IKK (IkB quinasa) en los residuos de serina 32 y 36. La fosforilación de IkB le confiere una elevada afinidad de sustrato por el sistema enzimático de conjugación de ubiquitina. La ubiquitinización convierte a IkB en un sustrato para el complejo proteolítico proteasoma. Así pues, la ubiquitinización controlada por fosforilación actúa provocando la degradación de IkB y por tanto liberando la forma activa de NF-kB. Esta situación de NF-kB activado es transitoria, ya que el mismo factor de transcripción regula positivamente la expresión génica de IkB [103]. Las especies reactivas, especialmente el H_2O_2, actúan como reguladoras de la actividad de este factor de transcripción [104]. Los mecanismos a través de los cuales las ROS modulan la actividad del NF-kB no están claros. La actividad IKK está regulada por fosforilación a través de componentes de la ruta de las MAPK, entre las que NIK (quinasa activadora de NF-kB) parece ser la más importante [105]. Como se ha mencionado anteriormente, las ROS pueden activar la ruta de las MAPK, lo que podría conducir a la fosforilación y activación de IKK. La activación de la cascada de MAPK iniciada por la activación de Ras por estrés oxidativo también puede provocar la fosforilación de una subunidad del propio NF-kB, lo que aumenta su actividad de transactivación

A pesar de que la inducción de las cascadas de señalización que conllevan la activación de factores de transcripción como el NF-kB y el AP-1 está potenciada por condiciones prooxidantes, la ejecución final de los procesos de señalización requieren condiciones relativamente reductoras. Tanto NF-kB como AP-1 presentan residuos conservados de cisteína en su región de unión al DNA, altamente susceptibles de ser oxidados y perdiendo así su capacidad de unión al DNA. Es evidente por tanto la necesidad de mantener un delicado equilibrio redox intermedio para poder conciliar estos requerimientos redox aparentemente contradictorios [106]. De esta forma se ha comprobado que para la activación de estos factores de transcripción se necesitan cantidades de ROS relativamente bajas, mientras que la presencia de niveles elevados de ROS conllevan la inhibición de la actividad de NF-kB y AP-1 [90].

El estrés oxidativo induce la expresión coordinada de toda una serie de genes entre los que se incluyen enzimas de detoxificación de fase II. Las regiones promotoras de muchos de estos genes contienen una secuencia denominada elemento de respuesta antioxidante (ARE). Algunos genes regulados por este elemento de respuesta son NADPH-quinona oxidoreductasa, glutatión-S-transferasa, UDP-glucuronosil transferasa, tioredoxina, hemooxigenasa-1 y ferritina [107]. Los factores de transcripción que se unen a estas regiones promotoras son Nrf1 y Nrf2.

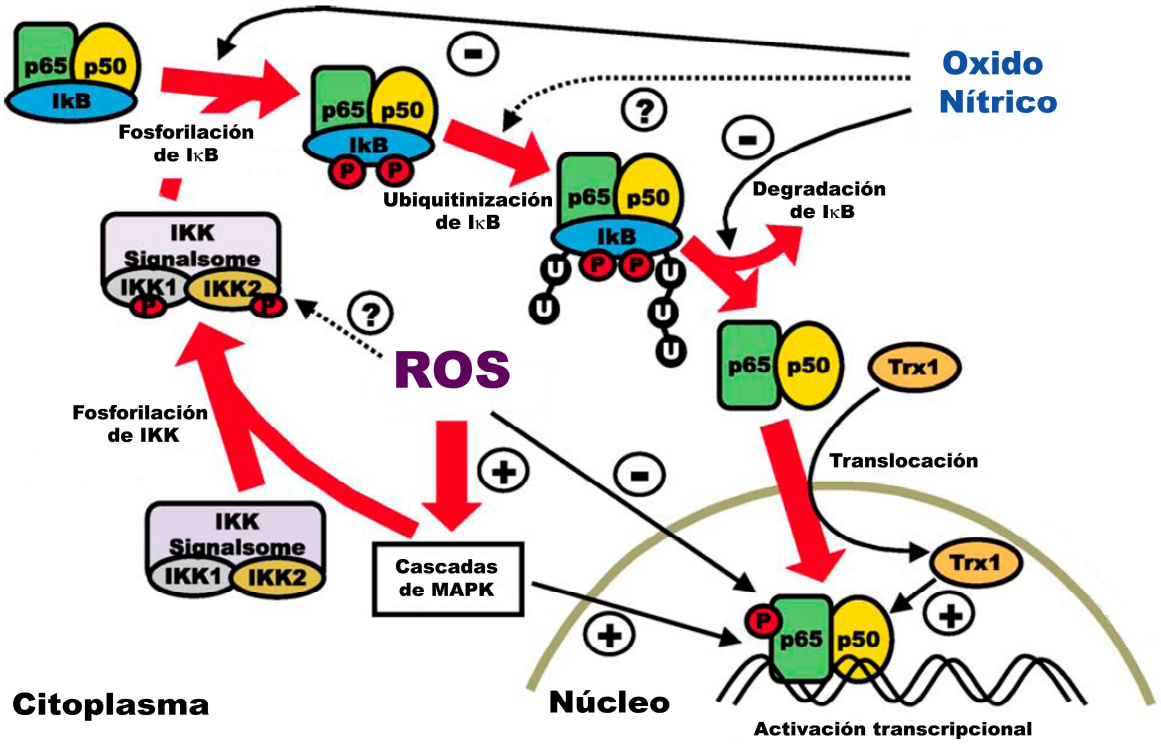

Figura 3. Mecanismo de activación y acción del NF-κB en respuesta a incrementos en los niveles de ROS. Adaptada de Jackson *et al*, 2002.

Nrf2 se puede encontrar secuestrada en el citoplasma por la proteína Keap1, asociada con el citoesqueleto, y su liberación permite la translocación al núcleo. De esta forma, la interacción entre NRf2 y Keap1 puede actuar como un sensor citoplasmático de estrés oxidativo [108]. Recientemente se ha descrito que la proteína quinasa CK2, una quinasa que en situaciones de estrés migra al núcleo donde aumenta su activada [109], podría ser la responsable de la fosforilación y activación de Nrf2 [110], aunque no se ha descrito un efecto directo de los ROS sobre la actividad CK2.

1.4.4. Efectos reguladores de las especies reactivas de nitrógeno

El óxido nítrico (NO) interviene en la regulación de la expresión de numerosos genes implicados en la respuesta inmune y la inflamación, interfiriendo en la activación del NF-kB. Esta regulación parece ser debida a las propiedades antioxidantes del NO, capaz de reaccionar con el $O_2^{\cdot-}$, y por tanto disminuyendo los niveles de intracelulares de ROS, activadores del NF-κB. Al ser la iNOS uno de los genes regulados por NF-κB, la presencia de altos niveles celulares de NO provocan una disminución de la activación del NF-κB, lo que se traduce en menor expresión de la iNOS y por tanto de

la producción de NO. Así pues, además de la inhibición directa que ejerce el NO sobre el grupo hemo de las óxido nítrico sintasas, el NO ejerce como modulador por retroalimentación negativa de la propia expresión de las NOS [103]. Una de las principales dianas del NO como señalizador celular es la guanilato ciclasa soluble (sGC), enzima que cataliza la conversión de guanosina trifosfato (GTP) a guanosina monofosfato cíclica (cGMP). De esta forma, una gran parte de los procesos de señalización celular asociados con el NO son llevados a cabo por el segundo mensajero cGMP, implicado en gran variedad de procesos fisiológicos en el sistema cardiovascular tales como la regulación del tono de la musculatura lisa vascular, la proliferación celular, la agregación plaquetaria y el reclutamiento de leucocitos [111, 112]. La reacción del NO con O_2 puede generar diversas especies que pueden reaccionar con grupos tiol, como los presentes en los residuos de cisteína de las proteínas, en un proceso denominado S-nitrosilación. La modificación de estos residuos de cisteína por parte de los óxidos de nitrógeno es otra de las vías por las que las RNS ejercen sus funciones reguladoras [113, 114]. A diferencia de los procesos de fosforilación y desfosforilación de proteínas, que se encuentran estrictamente regulados enzimáticamente, los procesos de S-nitrosilación no están sujetos a un grado de regulación tan estricto, y por tanto la especificidad de estos mecanismos es mucho menor [115].

El peroxinitrito es una especie reactiva implicada en el daño oxidativo detectado en varias condiciones patológicas. Es una especie con un elevado potencial oxidante que puede reaccionar de forma específica con determinados motivos proteicos clave en las vías de transducción de señal. El peroxinitrito es particularmente reactivo con los dedos de zinc (estructuras presentes en las regiones reguladoras de factores de transcripción y proteínas quinasas) y con centros ferrosulfurados (importantes en los componentes de la cadena respiratoria). Al reaccionar con los residuos de tirosina se forma 3-nitrotirosina, que no puede ser fosforilada. Esta nitración mimetiza la fosforilación y es capaz de activar ciertos enzimas, mientras que en otros enzimas la nitración bloquea su actividad. También es capaz de producir la nitración de proteínas del citoesqueleto afectando así a la translocación de señales celulares entre los compartimentos celulares [116]. Muchos factores de transcripción poseen residuos de cisteína imprescindibles para la unión al DNA. La oxidación de residuos de cisteína de factores de transcripción por parte del peroxinitrito puede tanto potenciar como inhibir la capacidad de unión de los factores de transcripción al DNA. Los factores de transcripción con dedos de zinc también son susceptibles de ser regulados por peroxinitrito. La estructura de dedos de zinc (compuesta por un átomo de zinc coordinado por residuos de cisteína e histidina) forma la estructura terciaria de

algunos factores de transcripción permitiendo el reconocimiento de regiones específicas de DNA. La oxidación del zinc-tiolato por parte del peroxinitrito provoca la liberación del átomo de zinc y la desestructuración del motivo, incapacitando la unión del factor al DNA. Otras proteínas que intervienen en la señalización celular tales como la proteína quinasa C (PKC) y Raf también poseen dedos de zinc susceptibles de ser oxidados por el peroxinitrito [116].

2. Células sanguíneas y especies reactivas de oxígeno

2.1. Eritrocitos

Los eritrocitos son células anucleadas y representan el tipo celular más abundante y uno de los más especializados del cuerpo humano. Su función principal es la de transportar el oxígeno (O_2) hacia los diferentes tejidos del organismo. Los eritrocitos se forman en la médula ósea, y al salir a la circulación pierden el núcleo, los ribosomas y las mitocondrias, por lo que pierden toda capacidad de división, síntesis de proteínas y reacciones oxidativas mitocondriales [117]. Más del 95% de la proteína citoplasmática del eritrocito es hemoglobina (Hb), una hemoproteína que permite la unión reversible del O_2 al grupo hemo. La membrana contiene un 40% de lípidos, incluye fosfolípidos y colesterol en un ratio molar 1.2:1 y aproximadamente la mitad de los ácidos grasos son insaturados. La glucosa es el único combustible que utiliza el eritrocito, y ésta es metabolizada a través de glucólisis anaeróbica. Los eritrocitos senescentes son finalmente retirados de la circulación por células fagocíticas, a un ritmo diario de aproximadamente el 1% de eritrocitos circulantes.

A pesar de no contener mitocondrias, y por tanto no producir ROS mitocondriales, los eritrocitos producen continuamente especies reactivas debido principalmente a la elevada tensión de O_2 en la sangre arterial y a su elevado contenido en hemo [118]. La principal fuente de ROS en el eritrocito es la hemoglobina, que puede ser autooxidada produciendo $O_2^{\cdot-}$. La reducción del O_2 a $O_2^{\cdot-}$ se acompaña de la oxidación de la hemoglobina a metahemoglobina (metHb), proteína que no posee capacidad de unión de O_2. La metahemoglobina puede ser reducida de nuevo a hemoglobina por el sistema NADH-citocromo b5-metHb reductasa y, en menor medida, por metHb reductasa NADPH-dependiente [119].

El $O_2^{\cdot-}$ generado es dismutado de forma casi inmediata a H_2O_2, una especie reactiva mucho más estable. El H_2O_2 puede provocar modificaciones oxidativas en la hemoglobina, que actuarán como señales para la proteólisis [120]. Además, el incremento en los niveles de H_2O_2 eritrocitarios se ha relacionado con incrementos en los niveles de metHb y peroxidación lipídica. El H_2O_2 puede a su vez reaccionar con el

$O_2^{\cdot-}$, dando lugar a $OH^{\cdot}$, molécula altamente reactiva. A pesar de que el estrés oxidativo eritrocitario puede dañar a la propia célula, la liberación de grandes cantidades de ROS al torrente sanguíneo también puede dañar otros componentes de la circulación [121]. La elevada proporción de ácidos grasos insaturados en su membrana, el ambiente rico en oxígeno y los abundantes niveles de Hb hacen de los eritrocitos un tipo celular especialmente susceptible de sufrir daño oxidativo.

Para combatir el estrés oxidativo, los eritrocitos contienen los enzimas SOD, catalasa, GPx y GRd y NADH-metHb reductasa. En el caso del eritrocito, al carecer de mitocondrias, la isoforma de SOD mayoritaria es la Cu/Zn-SOD citoplasmática. La presencia de niveles elevados de SOD permite la eliminación de radicales $O_2^{\cdot-}$ previniendo así la formación de metHb y del peroxinitrito, de forma que la actividad SOD también actúa como reguladora de la biodisponibilidad de óxido nítrico [122]. El H_2O_2 producido por la SOD puede ser detoxificado por la glutatión peroxidasa y por la catalasa. La GPx es la primera barrera contra el H_2O_2 en eritrocitos, detoxificándolo cuando éste se encuentra a bajas concentraciones. En cambio, cuando los eritrocitos se enfrentan a niveles incrementados de H_2O_2 la catalasa adquiere un papel predominante.

Junto con estos sistemas antioxidantes primarios que previenen la generación de radicales o la propagación de las reacciones en cadena, se ha descrito la presencia de otros sistemas secundarios, que incluyen proteasas que degradan selectivamente proteínas oxidadas. Una de éstas es una serina proteasa citoplasmática que en condiciones oxidantes se adhiere a la membrana celular y promueve la degradación de proteínas de membrana [123].

Los eritrocitos también poseen antioxidantes no enzimáticos como las vitaminas C y E, carotenoides y glutatión. Estudios recientes parecen indicar que los eritrocitos son portadores de glutatión por el torrente sanguíneo a partir de la síntesis de novo, proveyendo así un importante sistema de detoxificación en la circulación [124, 125]. El glutatión supone, de hecho, el antioxidante mayoritario en eritrocitos y protege proteínas eritrocitarias importantes como la espectrina, cuya oxidación puede provocar una mayor rigidez de las membranas, y mantiene los grupos sulfidrilo (SH) de la hemoglobina y otros enzimas en su estado reducido [126].

2.2. Linfocitos

Los linfocitos constituyen alrededor del 20-40% de los leucocitos totales y se pueden dividir en 3 poblaciones celulares en base a su función y sus componentes de membrana: linfocitos B, linfocitos T y células natural killer (NK). La función principal de los linfocitos es la de reconocer antígenos específicos y mediar la respuesta

inmunitaria específica o adquirida. Los linfocitos B son los que reconocen en primer lugar el antígeno extraño a través de anticuerpos específicos unidos a su membrana plasmática. Tras el reconocimiento del antígeno, el linfocito B se divide rápidamente para formar linfocitos B de memoria o células plasmáticas, cuya función es la de producir grandes cantidades de anticuerpo contra el antígeno reconocido. Los linfocitos T también pueden reconocer antígenos específicos, pero a diferencia de los linfocitos B sólo reconocen antígenos que se presentan junto a moléculas del complejo mayor de histocompatibilidad (MHC), que reconocen gracias a un receptor específico de estos linfocitos llamado receptor de células T (TCR). Entre los linfocitos T se distinguen tres subpoblaciones: linfocitos T cooperadores (T_H o CD4+), linfocitos T citotóxicos (T_C o CD8+) y linfocitos T supresores (T_S). Cuando un linfocito T reconoce un complejo MHC-antígenos comienza a secretar diferentes citoquinas cuya función será la de activar y regular la función de linfocitos B y T_C, macrófagos y otros células que participan en la respuesta inmune. Los linfocitos T_C son células efectoras que ejercen una función citotóxica sobre los patógenos extraños, encargándose de su eliminación. Las células natural killer (NK) son una porción minoritaria de linfocitos (aproxidamente el 5-10% de los linfocitos totales circulantes), y son células de mayor tamaño y con gránulos citoplasmáticos. Su función principal es la de eliminar células tumorales o infectadas con virus, en procesos tanto dependientes como independientes de anticuerpos.

La producción de ROS en el sistema inmunitario se ha estudiado principalmente en células fagocíticas como los macrófagos y los neutrófilos. Sin embargo, algunos estudios se han centrado también en la producción de ROS por parte de los linfocitos, y la importancia de estas ROS en la activación del linfocito frente a estímulos inmunitarios. Tras la estimulación de los linfocitos con agentes mitogénicos como el forbol miristato acetato (PMA) o concavalina A (ConA), así como con las señales que estimulan los receptores de las células T (TCR), se produce un incremento en la generación de ROS por parte de estas células [127, 128]. Entre las ROS producidas por el linfocito tras la estimulación de los TCR se encuentran el peróxido de hidrógeno y el anión superóxido, que parecen ser producidos por vías independientes [129]. Las vías a través de las cuales se generan estas especies reactivas en el linfocito han sido muy poco estudiadas hasta el momento, pero se postula que puedan ser similares a las de otros tipos celulares. El metabolismo del ácido araquidónico [130], la mitocondria [131] y la NADPH oxidasa [132] parecen ser algunas de las principales fuentes de ROS por el linfocito tras la activación del TCR. Al igual que ocurre en otros tipos celulares, las ROS producidas tras la estimulación de receptores de membrana parecen estar implicadas en procesos de señalización

celular. A pesar de que se han llevado a cabo pocos estudios sobre la regulación por especies reactivas en el linfocito, entre las dianas de las ROS generadas a este nivel parecen encontrarse las tirosina fosfatasas [128]. Tras la activación del receptor (TCR) comienza una cascada de fosforilaciones sucesivas reguladas por proteínas tirosina quinasas y fosfatasas que acaban con la formación del complejo de señalización. A partir de este complejo se activan diferentes vías de señalización, como la vía de las MAPK o la del NF-kB. En linfocitos, las vías de las MAPK están involucradas en la regulación del ciclo celular, mientras que el NF-kB participa en la regulación de la expresión de numerosos genes inflamatorios [133].

2.3. Neutrófilos

La función principal de los neutrófilos en la respuesta inmune innata es llevada a cabo a través de toda una seria de respuestas rápidas y coordinadas que culminan con la fagocitosis y eliminación de los patógenos [134]. Para ello, los neutrófilos contienen un potente arsenal antimicrobiano que consta de agentes oxidantes, proteinasas y péptidos antimicrobianos, y también producen grandes cantidades de ROS y RNS tales como $O_2^{-}\cdot$ y $NO^{\cdot}$ a través de la actividad de sistemas generadores de oxidantes como pueden ser la NADPH oxidasa y la óxido nítrico sintasa [135]. Durante la fagocitosis, el neutrófilo libera en el interior del fagosoma compuestos contenidos en diferentes gránulos (proteasas, fosfolipasas, glucosidasas y lisozimas) y ROS generados en la membrana del propio fagosoma, compartimentalizando así tanto al patógeno como a los productos citotóxicos y facilitando la eliminación del patógeno. A pesar de esta estructuración celular, en condiciones patológicas los compuestos citotóxicos pueden ser liberados al espacio extracelular e inducir daño en los tejidos circundantes [134].

Las principales fuentes de ROS y RNS en el neutrófilo son la NADPH oxidasa, la cadena de transporte electrónico, el metabolismo del ácido araquidónico y las óxido nítrico sintasas. La NADPH oxidasa (NOX) presente en los neutrófilos es un complejo enzimático unido a la membrana que, una vez activado, genera grandes cantidades de ROS [4], en lo que se conoce como la explosión oxidativa (del inglés *oxidative burst*). Cuando el neutrófilo está inactivo el enzima se encuentra latente, pero puede ser rápidamente activado por toda una serie de mediadores solubles (péptidos quimioatrayentes y quimioquinas) y determinados estímulos (bacterias y complejos inmunes) que interactúan con los receptores de membrana. Si bien el producto primario de la reacción es el $O_2^{-}\cdot$, éste puede formar rápidamente otras ROS tales como H_2O_2, $OH^{\cdot}$ y HOCl, y éstas especies reactivas serán liberadas en los fagosomas para oxidar las proteínas y lípidos bacterianos. La aportación de la cadena de

transporte electrónico a la producción de ROS en neutrófilos parece poco importante, ya que son un tipo celular que posee muy pocas mitocondrias.

El metabolismo del ácido araquidónico por las cicloxigenasas y lipoxigenasas también genera ROS. La prostaglandina H sintasa, enzima clave en la síntesis de prostaglandinas, prostaciclinas y tromboxanos, posee actividad cicloxigenasa e hidroperoxidasa, y ésta última es la responsable de la liberación de $O_2^{-}\cdot$. La 5-lipoxigenasa, responsables de la síntesis de leucotrienos, también produce $O_2^{-}\cdot$ [135]. Los neutrófilos también poseen óxido nítrico sintasa inducible (iNOS), enzima que produce $NO^{\cdot}$. El $NO^{\cdot}$ puede reaccionar con especies reactivas de oxígeno como el $O_2^{-}\cdot$ dando lugar a peroxinitrito, un compuesto potencialmente citotóxico pero que también funciona en la modificación fisiológica por nitrosilación de moléculas de señalización.

Para hacer frente a esta elevada producción de especies reactivas, los neutrófilos poseen un elaborado sistema de defensas antioxidantes, tanto enzimáticas (catalasa, glutatión peroxidasa, superóxido dismutasa) como no enzimáticas (vitaminas C y E, glutatión). Los neutrófilos contienen cantidades importantes de ascorbato, cuya función es la de preservar la integridad celular y la de del tejido circundante a través de la neutralización de los productos bactericidas producidos durante la explosión oxidativa [136, 137].

3. Situaciones fisiológicas o patológicas con riesgo de generar estrés oxidativo

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3.1. Hiperbaria, hiperoxia y buceo con botella

La hiperoxia es una condición en la cual la concentración de oxígeno se encuentra por encima de los niveles fisiológicos. La terapia con oxígeno hiperbárico (HBO) proporciona una presión de oxígeno del 100%, lo que provoca un incremento en la cantidad de oxígeno disuelto en la sangre y una disminución del tamaño de las burbujas de gas en sangre. Desde que se empezara a usar en 1939, esta terapia ha sido ampliamente utilizada en el tratamiento de diversas enfermedades, como la enfermedad de descompresión o la intoxicación por monóxido de carbono, y para favorecer la cicatrización de heridas y quemaduras [138-140]. Sin embargo, el aumento en los niveles de oxígeno se ha visto que a su vez conlleva un incremento en la concentración de ROS [141], y en la oxidación de lípidos, proteínas y ácidos nucleicos [142-144]. La respuesta celular al HBO se ha estudiado principalmente en modelos animales [145, 146], y sólo recientemente se ha comenzado a estudiar en humanos [147-149]. En todo caso, se ha sugerido que una sesión de HBO puede representar un buen modelo para la investigación del estrés oxidativo en humanos

[142, 150, 151]. Así, se ha detectado que una sesión de terapia hiperbárica en individuos sanos induce alteraciones importantes de las funciones de las células polimorfonucleadas (PMN) tales como un descenso de la quimiotaxis y un incremento en la producción de H_2O_2 y en la fagocitosis, así como un ligero incremento en el status antioxidante del plasma [152]. Así mismo, después de una sesión de terapia hiperbárica se detectó la aparición de daño oxidativo en las bases del DNA así como roturas en la cadena de DNA. Este daño a nivel de DNA se detectó sólo tras una primera sesión hiperbárica, pero no tras la aplicación de tratamientos sucesivos, indicando un incremento de las defensas antioxidantes y una respuesta adaptativa [142]. Otros estudios mostraron que células sanguíneas tomadas de individuos expuestos a HBO estaban completamente protegidas frente a la inducción de daño oxidativo en el DNA inducido por la administración de H_2O_2 [150]. Varios estudios del mismo grupo de investigación pusieron de manifiesto que esta respuesta adaptativa podía ser debida a la inducción del enzima hemooxigenasa-1 (HO-1) [149, 153], responsable de la degradación del grupo hemo, y cuyos productos de reacción son bilirrubina, ión ferroso y monóxido de carbono (CO). La HO-1 es un enzima altamente inducible por agentes que causan estrés oxidativo [154, 155], y parece tener un papel importante en el incremento de la resistencia celular contra el estrés y daño oxidativo [156-158]. Esta protección celular suele ser atribuida a la incrementada producción de bilirrubina, un reconocido antioxidante celular, y al incremento en el secuestro de hierro debido a los niveles aumentados de ferritina [154, 158].

El buceo con botella se caracteriza por la exposición a situaciones de hiperoxia resultantes del consumo de oxígeno a elevadas presiones típicas de la profundidad a la que se desciende. Junto con la situación de hiperoxia, durante la práctica del buceo se realiza una actividad física. Tanto la hiperoxia como la actividad física pueden conducir a una situación de estrés oxidativo, pero el posible estrés oxidativo asociado al buceo con botella no ha sido nunca estudiado hasta el momento.

3.2. Ejercicio

3.2.1. Estrés oxidativo asociado al ejercicio agudo

La práctica regular de ejercicio físico de intensidad moderada aporta efectos beneficiosos al organismo. Por el contrario, la práctica de episodios exhaustivos de ejercicio provoca daños estructurales a las células musculares y reacciones inflamatorias en el músculo. Algunos de estos efectos son consecuencia de una

producción elevada de especies reactivas de oxígeno provocada por la intensa actividad contráctil muscular.

Las principales fuentes de especies reactivas de oxígeno durante el ejercicio son la cadena de transporte electrónico mitocondrial, la xantina oxidasa y la NADPH oxidasa de macrófagos. La cadena de transporte electrónico mitocondrial produce ROS de forma continua en condiciones basales, en una proporción de aproximadamente el 2-4% del oxígeno consumido. Tradicionalmente se ha aceptado que por el mero hecho de que durante la práctica del ejercicio se consume más oxígeno, la producción de radicales también será mayor. Sin embargo, cuando la mitocondria entra en estado 3 para producir grandes cantidades de ATP, con el consiguiente incremento en el flujo electrónico, la proporción de oxígeno que es convertido a radicales libres cae hasta aproximadamente el 0,25%, lo que supone una orden de magnitud menos que en condiciones basales [159, 160]. En esta menor producción de ROS podrían estar implicadas las proteínas desacoplantes UCP-2 y UCP-3, que actuarían protegiendo a la mitocondria frente a la producción de ROS y la aparición de daño celular [161]. Recientemente se ha propuesto un mecanismo para explicar un posible aumento de la producción mitocondrial de ROS según el cual las elevadas temperaturas que alcanza el interior del músculo durante la actividad física (entre 41 y 45ºC) provocan una desestabilización de la ubisemiquinona, lo que induce un descenso en la transferencia electrónica a lo largo de la cadena a la vez que la transferencia directa del electrón al oxígeno aumenta [162-164]. En todo caso, a día de hoy el papel de la cadena respiratoria como fuente de ROS durante el ejercicio es objeto de debate [165].

La xantina oxidoreductasa se puede encontrar en dos isoformas con actividad diferente, xantina oxidasa (XO) y xantina deshidrogenasa (XDH). En condiciones fisiológicas normales, la forma predominante es la XDH, que transfiere el electrón al NAD, dando lugar a NADH. Sin embargo en condiciones temporales de hipoxia o isquemia predomina la actividad XO, que reduce el oxígeno hasta superóxido y peróxido de hidrógeno. Los resultados obtenidos por diferentes grupos de investigación apuntan hacia un papel importante de la XO en la generación de ROS durante el ejercicio y la posterior inducción de daño oxidativo. La administración de alopurinol, un inhibidor del enzima, previno la aparición de glutatión oxidado y contra el incremento plasmático de creatina quinasa, aspartato aminotransferasa y malondialdehído después de una sesión de ejercicio en humanos [166, 167].

Recientemente se ha centrado la atención en otro posible mecanismo de producción de especies reactivas durante la práctica del ejercicio, especialmente si este es exhaustivo, excéntrico o prolongado. Este mecanismo consiste en la

producción de radicales por parte de los neutrófilos y otras células fagocíticas que ejercerían una respuesta inmune al daño en los tejidos generado por los episodios de ejercicio intenso o de larga duración. De esta forma, los neutrófilos migran hacia los tejidos dañados para fagocitar las células dañadas, liberando $O_2^{-}\cdot$ y lisozimas [168]. La activación de los neutrófilos y la respuesta inmune al ejercicio se explican con más detalle en el apartado 4.2.3.

Aunque las fuentes de ROS no están del todo caracterizadas, de lo que no cabe duda es que el exceso de ROS producido durante la práctica de actividad física intensa o prolongada resulta en la oxidación de biomoléculas y en la aparición de estrés oxidativo. Estudios muy tempranos ya detectaron la presencia de niveles incrementados de pentano oxidado después de una sesión de ejercicio prolongado [169]. Desde entonces, y hasta día de hoy, numerosos estudios han puesto de manifiesto incrementos en los marcadores de peroxidación lipídica después de la práctica de actividad física intensa, e incluso se han establecido relaciones entre los niveles de peroxidación lipídica (midiendo principalmente los niveles de MDA) y la intensidad del ejercicio [46, 170-172]. A pesar de que la oxidación de proteínas por parte de las ROS está ampliamente descrita, hasta hace relativamente poco no se ha empezado a determinar la oxidación de proteínas producida durante el ejercicio físico. Se han documentado incrementos en los niveles de grupos carbonilos después de una carrera de maratón [173], una etapa ciclista [174], una sesión de ejercicio de intesidad submáxima (al 70% de la VO_2max) [175, 176] y una sesión de ejercicio de intensidad máxima [177]. Finalmente también se ha comprobado que la práctica de actividad física intensa produce daño oxidativo en el DNA, midiendo tanto las roturas de la cadena por el método del comet assay [178-180] como los niveles de 8-hidroxideoxiguanosina (8-OHdG) como marcadores de oxidación de las bases del DNA [181-183].

Es pues evidente que la práctica de actividad física de forma esporádica y sobretodo si es de elevada intensidad o de larga duración conduce a la generación de niveles elevados de especies reactivas de oxígeno, y éstas a la oxidación y modificación de los diferentes componentes celulares. Para poder hacer frente a estos episodios hiperoxidativos, se ha puesto de manifiesto que las células adaptan sus defensas antioxidantes. Los niveles de vitamina E en los tejidos no suelen variar como consecuencia de una sesión de ejercicio, lo que sugiere que los niveles fisiológicos de esta vitamina en los tejidos son los adecuados para proteger contra la incrementada producción de ROS asociada al ejercicio [184, 185]. El glutatión es una de las defensas antioxidantes principales a la hora de hacer frente al estrés oxidativo generado por la actividad física. Durante el ejercicio, grandes cantidades de GSH son

oxidadas a GSSG en el músculo y en el corazón para neutralizar las especies reactivas y productos de oxidación. Sin embargo, el ratio GSH:GSSG no suele llegar a alterarse significativamente debido a los mecanismos enzimáticos de regeneración de GSH y al importe de GSH desde el plasma vía ciclo del g-glutamilo [186-188]. Los enzimas antioxidantes pueden activarse de forma selectiva durante una sesión de ejercicio extenuante en función de la situación de estrés oxidativo impuesta en el tejido así como de la capacidad antioxidante del mismo. El músculo esquelético necesita una mayor protección antioxidante contra el potencial daño oxidativo que ocurre durante o después del ejercicio. Se han descrito incrementos en la actividad de los enzimas catalasa, superóxido dismutasa (SOD) y glutatión peroxidasa (GPx) en respuesta al ejercicio. Así se ha visto que un episodio agudo de actividad física es suficiente para incrementar la actividad SOD en diferentes tejidos incluyendo el hígado, músculo esquelético y eritrocitos [17, 189], sin embargo la regulación de la actividad SOD varía en función de las diferentes isoformas del enzima (Cu/Zn-SOD y Mn-SOD) y de los diferentes tipos de ejercicio [17]. La actividad GPx, en cambio, ha mostrado respuestas variables al ejercicio en función del tipo de músculo esquelético. De esta forma se ha descrito tanto que la actividad de este enzima no varía tras realizar ejercicio [190, 191] como que aumenta [192].

Se ha descrito recientemente que diferentes rutas de señalización celular son activadas durante o tras la realización de ejercicio, y la activación de estas rutas podría estar relacionada con la activación de las defensas antioxidantes. Así, se ha detectado tras una sesión de ejercicio la activación simultánea del factor de transcripción NF-kB y la expresión génica de la Mn-SOD en músculo esquelético de rata [193, 194], la activación de la vía de las MAPK (p38, ERK1 y ERK2), activación del NF-kB y aumento en la expresión de genes como la SOD y la iNOS en músculo de rata [195], y la activación de la subunidad p50 del NF-kB en linfocitos humanos [196]. El hecho de que el alopurinol (inhibidor de la producción de ROS por parte de la xantina oxidasa) inhibiera estas respuestas adaptativas apunta a las ROS como principales efectoras.

3.2.2. El entrenamiento como antioxidante

Si bien un episodio puntual de ejercicio intenso y/o prolongado puede generar daño oxidativo y conducir a una situación de estrés oxidativo, la práctica regular de actividad física moderada potencia las defensas antioxidantes y aporta una mayor resistencia a la aparición de estrés oxidativo. Junto con esta potenciación de las defensas, está ampliamente documentado que la práctica de ejercicio moderado otorga una mayor protección frente a diferentes enfermedades y, en general,

promueve un mejor estado de salud. La explicación a este efecto positivo de la práctica regular del deporte podría ser el fenómeno conocido como hormesis, según el cual la presencia continua de pequeños estímulos, tales como bajas concentraciones de ROS, podría inducir la expresión de enzimas antioxidantes y otros mecanismos de defensa [197-199]. La hormesis es, en toxicología, un fenómeno de dosis respuesta caracterizado por la estimulación a bajas dosis e inhibición a altas dosis, que resulta en una curva no monotónica (en forma de U invertida) [197].

El efecto del entrenamiento físico sobre las defensas antioxidantes y el estrés oxidativo se ha estudiado principalmente en el músculo. Se ha comprobado que animales que han sido entrenados muestran menores niveles de daño oxidativo tras la realización de ejercicio extenuante o tras la inducción de estrés oxidativo en comparación con animales no entrenados [200-203]. Los enzimas implicados en la reparación del DNA también responden de forma positiva al entrenamiento, lo que se traduce en una mayor resistencia a la aparición de lesiones en el DNA [203, 204]. Debido a que la respuesta adaptativa resulta de los efectos acumulativos de los episodios repetidos de actividad física, la señal inicial para la estimulación que lleva a la modulación a largo plazo debe ocurrir después de cada sesión individual [205].

En cuanto a los enzimas antioxidantes, se ha comprobado que los individuos entrenados suelen poseer, por lo general, mayores actividades que los individuos no entrenados. La actividad SOD se ha visto incrementada en el músculo esquelético en respuesta a diferentes tipos de entrenamiento, pero a la vez estudios diferentes no detectaron modificaciones [17]. Las discrepancias podrían ser debidas a las diferentes isoformas estudiadas y los métodos de determinación utilizados, a los diferentes modelos de entrenamiento y a diferentes tipos de fibras usadas. Se ha descrito que la actividad Cu/Zn-SOD aumenta sin incrementos asociados de los niveles de proteína ni de la expresión génica. En el caso de la Mn-SOD, por el contrario, se ha descrito que el incremento en la actividad va acompañado de un incremento en los niveles proteicos, pero no así de expresión. Estos resultados sugieren que la inducción por entrenamiento de ambas isoformas de SOD está causada por mecanismos post-transcripcionales, y que la modulación post-traduccional podría jugar un papel importante en la activación de la Cu/Zn-SOD [17]. Numerosos estudios han puesto también de manifiesto la inducción de actividad glutatión peroxidasa en músculo esquelético tras un período de entrenamiento [206-209]. Esta adaptación también parece ser específica del tipo de fibra muscular, con las fibras tipo 2a como las que más responden [210]. Finalmente, aún no está clara la respuesta de la catalasa al entrenamiento, ya que hasta el momento se han obtenido resultados contradictorios al respecto, documentándose tanto incrementos como descensos, así como también

carencia de efectos [17]. La razón por la cual los diferentes enzimas e isoformas no responden por igual al entrenamiento no está clara, pero podría depender del patrón específico de expresión génica así como de los umbrales requeridos para la inducción de la actividad o expresión. La síntesis de novo de un enzima requiere una gran cantidad de energía y es un proceso lento, por lo que este mecanismo suele estar reservado hasta la máxima necesidad. La actividad SOD es relativamente alta e uniforme en los diferentes tejidos del organismo, lo que parece indicar que la eliminación del anión superóxido no es un paso limitante en la detoxificación de las ROS. Por el contrario, la actividad GPx, que se encarga de eliminar productos finales de las ROS tales como peróxidos lipídicos, presenta actividades relativamente menores, lo que podría explicar el hecho de que la glutatión peroxidasa muestra una mayor adaptación al entrenamiento que otros enzimas antioxidantes como la SOD y la catalasa [17].

3.2.3. Ejercicio y sistema inmune

3.2.3.1. Respuesta inmune de fase aguda

La respuesta de fase aguda (RFA) es la parte de la respuesta inmunitaria inespecífica, es decir, que no depende del reconocimiento de antígenos concretos. En esta respuesta están implicadas las células del sistema inmunitario pero también células del sistema nervioso central, hígado, páncreas, riñón y músculo esquelético. La respuesta de fase aguda incluye fiebre, leucocitosis, redistribución de hierro de compartimentos extracelulares a intracelulares y producción hepática acelerada de determinadas proteínas plasmáticas, todo ello destinado a dificultar el crecimiento y supervivencia de los patógenos en el organismo huésped [211]. Los leucocitos activados fagocitan los microorganismos infecciosos, y las proteínas plasmáticas de fase aguda tales como la proteína C reactiva opsonizan las bacterias y los restos celulares de forma que potencian la fagocitosis [212]. Junto con esto, la producción de grandes cantidades de ROS por parte de los neutrófilos y los monocitos es también un mecanismo implicado en la destrucción de los patógenos invasores [213]. En la modulación de la RFA intervienen diferentes citoquinas, principalmente interleuquina (IL)-1, IL-6 y el factor de necrosis tumoral (TNF) [214]. Los microorganismos patógenos y fragmentos celulares de tejidos dañados, así como el sistema del complemento y las especies reactivas producidas por el organismo huésped, estimulan a los monocitos y los macrófagos a producir estas citoquinas.

Tras una cascada de activaciones proteolíticas del sistema del complemento, el número de neutrófilos circulantes aumenta drásticamente en pocas horas, y éstos migran rápidamente al lugar de la infección, donde fagocitan los microorganismos patógenos y los restos celulares [137]. De forma más lenta (en cuestión de días en lugar de horas) los monocitos también alcanzan el sitio de la infección y contribuyen a la eliminación de los patógenos y restos celulares, pero también proporcionan los factores que promueven la reparación y regeneración del tejido [215]. De los tres tipos de células fagocíticas, los neutrófilos son los que producen mayores cantidades de ROS, seguidos de los monocitos, y finalmente los macrófagos como los que producen menores cantidades [213]. Tras la unión de estos macrófagos a diferentes factores solubles o particulados se desata la explosión oxidativa. El primer paso consiste en el ensamblaje de las subunidades de la NADPH oxidasa, enzima que produce aniones superóxido a través de la transferencia de electrones desde el NADPH al O_2. Este $O_2^{-}\cdot$ puede ser dismutado a H_2O_2 (con mayor actividad bactericida) o, en presencia de mieloperoxidasa (enzima almacenado en los gránulos azurófilos de los neutrófilos), dar lugar a ácido hipocloroso. Las diferencias antes mencionadas en cuanto a la capacidad de producción de ROS de los diferentes fagotitos son paralelas a diferencias relativas en los niveles celulares de mieloperoxidasa [213]. La mayoría de estas ROS son secretadas en el interior de los fagosomas, pero se puede producir cierta pérdida al medio extracelular, lo cual puede causar daño celular en los tejidos objeto de la infección.

Una parte importante de la RFA es la inducción de defensas antioxidantes producida por las diferentes citoquinas. Así, los niveles de Mn-SOD, catalasa, ceruloplasmina, óxido nítrico sintasa y metalotioneína incrementan en respuesta a las interlequinas IL-1 e IL-6 y el TNF [216-218]. Los efectos de las citoquinas sobre regulación de las especies reactivas de oxígeno se resumen en la Tabla 1.

Tabla 1. Regulación de la producción y eliminación de especies reactivas de oxígeno mediada por citoquinas.

Citoquina	Acción
Interferón, TNF	Incremento de la producción de radicales de oxígeno
IL-1, TNF	Incremento de los niveles de Mn-SOD, metalotioneína, catalasa
IL-6	Incremento de la síntesis hepática de ceruloplasmina
IL-1	Promoción del secuestro de hierro
IL-1	Inducción de la óxido nítrico sintasa

Junto con su papel como efectores de la destrucción de patógenos, las ROS también participan en la RFA como moduladores de otros mecanismos efectores. Diferentes enzimas proteolíticos son almacenados en forma latente y activados en presencia de ROS [219]. De la misma forma, las ROS promueven la activación del complemento [220] y facilitan la translocación vascular de leucocitos a través de la inducción de la expresión de moléculas de adhesión [221].

3.2.3.2. Respuesta de fase aguda asociada al ejercicio

La realización de una sesión de ejercicio físico intenso conlleva una secuencia de eventos similar a la respuesta de fase aguda inducida por una infección, pero de una magnitud menor. El sistema del complemento se ve activado tras el ejercicio a través de la denominada vía alternativa, que puede ser estimulada por fragmentos de tejido dañado [222-224]. La leucocitosis (principalmente neutrofilia) es otra de las caracterísiticas típicas de la respuesta inmunitaria al ejercicio y se describió hace ya más de 100 años tras una maratón [225]. Posteriormente se comprobó que ejercicios mucho más cortos también son capaces de inducirla [226, 227]. Esta neutrofilia asociada al ejercicio parece ser debida principalmente al daño muscular y la consecuente activación del complemento. A parte de incrementar notablemente su número en circulación, los neutrófilos también se activan durante el ejercicio, lo que se ha determinado *in vivo* a través de la determinación de concentraciones plasmáticas elevadas de enzimas almacenados normalmente en los gránulos del neutrófilo (elastasa [228], lactoferrina [229] y mieloperoxidasa [230]) e *in vitro* por una mayor producción de ROS por parte de neutrófilos aislados tras el ejercicio [231]. Los neutrófilos se infiltran rápidamente en el tejido muscular dañado, y la magnitud de esta infiltración es proporcional al grado de daño en las bandas z [232]. Los monocitos también migran a las regiones dañadas del músculo tras el ejercicio y, al igual que en la respuesta de fase aguda a la infección, lo hacen mucho más tarde que los neutrófilos. Es importante remarcar que estas células aparecen después de la aparición de daño, por lo que la acción de los fagocitos más que causar daño es la que promueve la reparación del tejido dañado. En modelos de daño muscular en rata se identificaron diferentes subpoblaciones de macrófagos en las etapas tempranas de fagocitosis en comparación con las etapas de recuperación muscular posteriores [233].

La producción de citoquinas, especialmente IL-1 e IL-6, también se ve modulada por la práctica de ejercicio. Diferentes ensayos tanto in vitro como in vivo han puesto de manifiesto incrementos tanto en la expresión como en la secreción de IL-1 e IL-6 [234-236]. El daño muscular no parece ser el responsable, o requisito, para

la liberación de interleuquinas. Entre los factores que se han postulado como inductores de esta producción de citoquinas se encuentran los lipopolisacáridos producidos por el tracto gastrointestinal, la fagocitosis de fragmentos de tejido y los peróxidos lipídicos producidos por las ROS [137].

3.2.3.3. Adaptación de las células sanguíneas al ejercicio

Las diferentes células sanguíneas han demostrado ser tipos celulares adecuados para estudiar los efectos del ejercicio sobre el estrés oxidativo. Debido al incremento en el consumo de oxígeno asociado a la actividad física, los eritrocitos deben transportar mayores cantidades de oxígeno a los tejidos, lo que les hace especialmente susceptibles de padecer estrés oxidativo (apartado 3.1). Diferentes estudios han puesto de manifiesto que a pesar de ser células que no tienen la capacidad de síntesis de nueva proteína, los eritrocitos son capaces de adaptar sus defensas antioxidantes en respuesta al estrés oxidativo asociado al ejercicio por mecanismos de regulación post-traduccional. Tras una carrera de duathlon las actividades catalasa y glutatión peroxidasa aumentaron en eritrocitos [237, 238]. Tras una etapa ciclista de montaña se observó también un incremento en la actividad catalasa y glutatión reductasa, pero a la vez un descenso en la actividad glutatión peroxidasa [239]. Sin embargo, la activación de las defensas observada en estos estudios parece no ser suficiente para contrarrestar por completo el estrés oxidativo, puesto que tras una etapa ciclista de montaña se detectó un aumento en los marcadores de daño oxidativo en lípidos y proteínas [240].

Como se ha descrito anteriormente, los neutrófilos participan activamente en la respuesta de fase aguda al ejercicio, activándose durante el proceso y produciendo grandes cantidades de especies reactivas de oxígeno en la explosión oxidativa. Se ha comprobado que este incremento en el potencial oxidativo del neutrófilo tras un ejercicio intenso va acompañada de un descenso en la actividad de los enzimas antioxidantes catalasa, SOD y GPx, así como en los niveles de glutatión total y del ratio GSSH:GSSG y un incremento en los niveles intracelulares de ascorbato [241, 242]. Tras una etapa ciclista de montaña se observó también una marcada neutrofilia así como una disminución de las actividades catalasa y glutatión peroxidasa en neutrófilos, pero estas menores actividades enzimáticas no se vieron acompañadas de la aparición de daño oxidativo en proteínas [240]. El neutrófilo podría actuar por tanto como una célula que aportara sus defensas antioxidantes al plasma sanguíneo para protegerlo así de la posible aparición de estrés oxidativo. A pesar de quedar en cierta

medida indefenso por esta pérdida de antioxidantes, el neutrófilo parece ser resistente a padecer estrés oxidativo.

Los linfocitos muestran una respuesta al ejercicio opuesta a la de los neutrófilos. El número de linfocitos aumenta durante la realización del ejercicio para luego caer drásticamente hasta niveles inferiores a los iniciales [243]. Los enzimas antioxidantes tienden a aumentar su actividad tras la práctica de actividad física intensa [242], pero esta respuesta es dependiente de la duración e intensidad del ejercicio. Así, tras una prueba de esfuerzo máximo pero de corta duración el número de linfocitos aumentó y las actividades linfocitarias catalasa y glutatión peroxidasa disminuyeron, mientras que tras una prueba de esfuerzo submáximo de mayor duración no se observó linfocitosis y la actividad glutatión peroxidasa aumentó [244]. Estos resultados apuntan a una posible inactivación temprana de los enzimas antioxidantes como consecuencia del incremento de los niveles de ROS, pero que luego se ve compensada con un incremento en los niveles o la actividad de estos enzimas si el estímulo persiste. En línea con este modelo, tras una etapa ciclista de montaña se observó la activación de los enzimas catalasa, glutatión peroxidasa, glutatión reductasa y superóxido dismutasa, pero la activación de estas defensas no fue suficiente para contrarrestar la aparición de daño oxidativo [240, 245]. La superóxido dismutasa parecer ser uno de los enzimas implicados en la adaptación al estrés oxidativo asociado al ejercicio en el linfocito, tal como parece ser en el músculo esquelético. Tras una etapa ciclista que no incluía tramos de montaña (por lo que la intensidad del ejercicio es menor que en una etapa de montaña) se detectó únicamente la potenciación de la actividad SOD pero no del resto de enzimas antioxidantes, y esta potenciación era debida, al menos en parte, a la activación de la expresión génica tanto de la Cu/Zn-SOD como de la Mn-SOD [246]. Estos resultados apuntan a que la respuesta adaptativa del linfocito al ejercicio puede ser similar a la observada en otros tejidos como el músculo esquelético. Sin embargo, no se debe descartar por ello la presencia de otros mecanismos reguladores.

3.3. Porfiria variegata

3.3.1. El grupo hemo

El grupo hemo es esencial para la vida y, a pesar de ser sintetizado en todas las células nucleadas, se sintetiza mayoritariamente en el hígado y la médula ósea. Un esquema de la ruta de síntesis del grupo hemo se muestra en la Figura 4. La configuración única de la estructura tetrapirrólica del hemo, con sus dobles enlaces

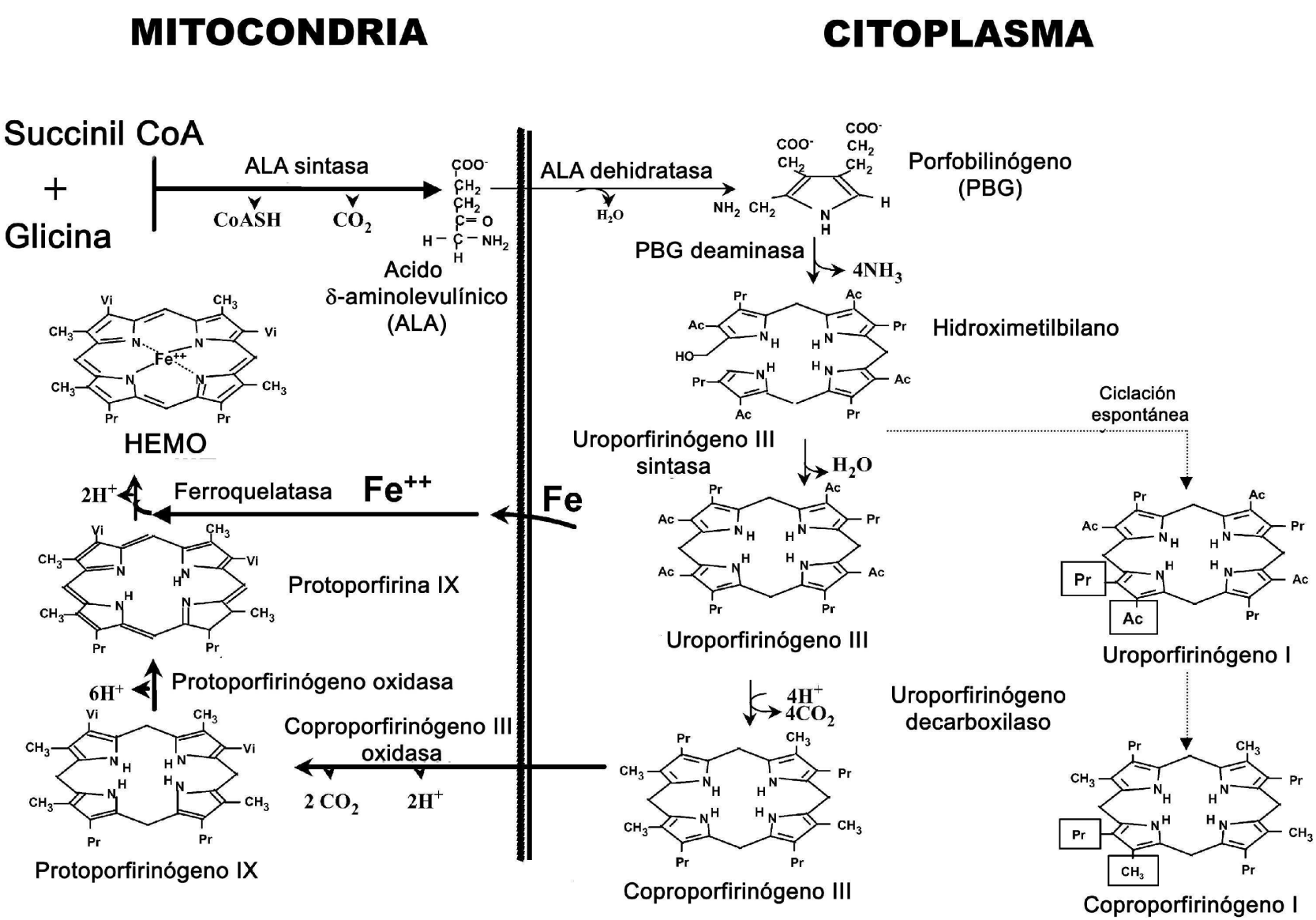

Figura 4. Ruta de biosíntesis del grupo hemo.

dispuestos simétricamente alrededor de un átomo de hierro central, permite su función en un gran abanico de reacciones vitales como portador de electrones, catalizador de reacciones redox y portador de oxígeno. Tras su síntesis en la membrana mitocondrial interna, el hemo es translocado a la mitocondria, microsomas, peroxisomas, citoplasma y núcleo. Más de la mitad de la producción hepática de hemo se usa para la formación de citocromos P450, implicado en el metabolismo de esteroides y en el metabolismo oxidativo de compuestos lipofílicos externos, incluyendo disolventes orgánicos y medicamentos. Para la obtención de energía, todas las células nucleadas del cuerpo sintetizan citocromos respiratorios mitocondriales. El grupo hemo también forma parte de la catalasa, enzima implicado en la descomposición del peróxido de hidrógeno, y de la guanilato ciclasa, enzima que tras ser activado por óxido nítrico participa en la relajación de los vasos sanguíneos y en numerosas reacciones fisiológicas. Es por tanto evidente que interferencias en la producción de hemo puedan tener consecuencias para el organismo relacionadas con la provisión de energía, resistencia al estrés oxidativo y el potencial detoxificador de medicamentos, así como con la función de diferentes vías de transmisión de señal.

3.3.2. La porfiria

Las porfirias son un grupo de enfermedades metabólicas que resultan de desórdenes en la biosíntesis del grupo hemo [247]. Cada tipo de porfiria es el resultado de la disminución en la actividad de un enzima concreto en la cadena de síntesis del grupo hemo, y se ha detectado malfuncionamiento enzimático en siete de los ocho enzimas que conforman esta cadena de síntesis. La dificultad por acabar la ruta de síntesis conlleva la acumulación de metabolitos intermediarios como pueden ser las porfirinas y sus precursores. La acumulación de estos precursores se asocia con toda una serie de manifestaciones clínicas características de cada porfiria y también permite el diagnóstico adecuado tras la determinación de dichos precursores en plasma, orina, heces y eritrocitos [248].

La situación basal es por regla general asintomática, pero ciertos desencadenantes ambientales producen crisis porfíricas. Algunos medicamentos (barbitúricos, estrógenos), ayuno, tensión emocional y física, infecciones, consumo de alcohol y drogas (marihuana, éxtasis, anfetaminas y cocaína) y situaciones cíclicas como la menstruación son factores que pueden desencadenar los ataques agudos de porfiria. Cuando se da un ataque agudo, el tratamiento requiere de glucosa oral e intravenosa y hematina o arginato de hemina [249] como los principales fármacos. Éstos actúan reduciendo los niveles del ácido aminolevulínico al inhibir su síntesis,

dando como resultado una remisión clínica y bioquímica del episodio agudo, momento a partir del cual la excreción urinaria de ácido aminolevulínico y porfobilinógeno tiende a normalizarse. La terapia con hemina provoca un aumento de la función del citocromo P450 en hígado durante la manifestación de crisis agudas de porfiria [249]. De esta manera la terapia con arginato de hemina no sólo suprime la superproducción de los precursores del grupo hemo sino que también mejora el metabolismo del oxígeno durante los ataques agudos de porfiria.

En España no se tienen datos oficiales de la incidencia, sólo se dispone de datos estimativos que ya se encuentran sobrepasados debido a las recientes campañas de sensibilización e información llevadas a cabo por la Asociación Española de Porfirias. El número total de casos previos a la campaña informativa era de 1427, de los que en el área mediterránea se recogen el 77% de los casos. Las últimas estimaciones apuntan a una prevalencia de 1,24 casos por 1000 habitantes [250]. La edad media estimada de los afectados de porfiria en España es de 30 años, siendo el 88% mujeres.

3.3.3. Clasificación de las porfirias

Las porfirias se pueden clasificar en dos grandes grupos en función del órgano en el que se produce principalmente el exceso de producción de porfirinas o sus precursores. Así se distinguen las porfirias hepáticas y las porfirias eritropoyéticas. Otra clasificación se basa en los síntomas clínicos y así podemos diferenciar las porfirias agudas de las porfirias cutáneas. Se han descrito cuatro tipos distintos de porfirias hepáticas agudas: porfiria aguda intermitente (PAI), coproporfiria hereditaria (CH), porfiria variegata (PV) y porfiria por deficiencia de δ-aminolevulinato (ALA) dehidratasa (PDA). Todas las manifestaciones clínicas de un ataque agudo se pueden explicar por las lesiones producidas en diferentes áreas y componentes del sistema nervioso central y periférico [251, 252]. Los ataques agudos, asociados a la superproducción de porfobilinógeno (PBG) y ALA, normalmente comienzan con dolor abdominal generalizado, diarrea, náuseas y vómitos. Estos ataques agudos se observan mayoritariamente en mujeres de entre 18 y 40 años [253] y es frecuente que se intensifiquen de forma recurrente durante la semana previa a la menstruación. En algunos casos pueden aparecer trastornos mentales tales como ansiedad, depresión, desorientación o alucinaciones [254]. Estos trastornos suelen desaparecer durante la remisión del ataque y no conforman una enfermedad psiquiátrica [255]. A menudo se hace complicado distinguir la PAI de la CH o la PV. Cuando se producen simultáneamente ataques agudos y lesiones cutáneas (similares a las presentes en la

porfiria cutanea tarda, PCT) se puede descartar la PAI, puesto que ésta nunca presenta lesiones cutáneas. Sin embargo, en la PV las lesiones cutáneas pueden producirse con anterioridad a los ataques agudos, y entonces es muy difícil distinguirla de la PCT. En la CH las lesiones cutáneas se dan con menos frecuencia que en la PV, y por tanto es mucho más difícil distinguirla de la PAI.

Tabla 2. Clasificación de las porfirias

Porfiria	Enzima	Herencia	Clínica
Hepáticas			
Porfiria por deficiencia de ALA dehidratasa (PDA)	ALA dehidratasa	AR	Aguda
Porfiria aguda intermitente (PAI)	Hidroximetilbilano sintasa	AD	Aguda
Porfiria cutanea tarda (PCT)	Uroporfirinógeno decarboxilasa	Esporádica (80%) AD (20%)	Cutánea
Coproporfiria hereditaria (CH)	Coproporfirinógeno oxidasa	AD	Aguda ± cutánea
Porfiria variegata (PV)	Protoporfirinógeno oxidasa	AD	Aguda ± cutánea
Eritropoyéticas			
Porfiria eritropoyética congénita (PEC)	Uroporfirinógeno III sintasa	AR	Cutánea
Protoporfiria eritropoyética (PPE)	Ferroquelatasa	AD	Cutánea

ALA, ácido δ-aminolevulínico

3.3.4. Síntomas y tratamiento de las porfirias

En el manejo de los ataques agudos lo primero que se debe hacer es identificar y eliminar los factores precipitadotes del ataque y tratar los síntomas con analgésicos, antieméticos y ansiolíticos. Tras el tratamiento sintomático se suelen utilizar dos terapias específicas: glucosa y hematina. La administración de glucosa normalmente conduce a una reducción en la excreción urinaria de precursores de porfirinas. Un suplemento adecuado (300-400 g/día) se suele administrar por perfusión intravenosa. Tras la introducción de la hematina como terapia, el tratamiento de los ataques porfíricos se ha visto notablemente mejorado. La hematina también se administra de forma intravenosa en dosis de hasta 3-4 mg/kg/día durante 4 días. Se ha comprobado

que el hemo accede hasta el hepatocito y reabastece el acervo de hemo, disminuyendo la actividad ALA sintasa. La investigación de antecedentes familiares y las recomendaciones preventivas son muy importantes a la hora de identificar a los pacientes presintomáticos e informarlos sobre cómo se puede limitar el riesgo de sufrir ataques agudos con simples modificaciones de los hábitos de vida como evitar el consumo de alcohol y de ciertos medicamentos [256].

Aproximadamente el 80% de los individuos que heredan un gen para una de las porfirias autosómicas dominantes permanecen asintomáticos durante toda la vida, y un 66% de los porfíricas latentes presentan excreción normal de precursores y porfirinas [257].

3.3.5. Porfiria variegata

La porfiria variegata es una porfiria hepática, autosómica dominante, de baja penetrancia (hasta un 75% de portadores asintomáticos) y caracterizada clínicamente por lesiones dérmicas y ataques agudos que pueden ocurrir separados o de forma simultánea. Resulta de la deficiencia parcial del penúltimo enzima de la ruta de biosíntesis del hemo, la protoporfirinógeno oxidasa (PPOX, [E.C.1.3.3.4]), responsable de la oxidación del protoporfirinógeno IX a protoporfirina IX. La actividad PPOX en los pacientes heterozigotos está reducida hasta aproximadamente la mitad de los niveles normales [258]. Esta deficiencia viene causada por mutaciones en el gen PPOX, de 5,5 kb de tamaño y localizado en el cromosoma 11 22-23 [259]. La PPOX humana es una flavoproteína homodimérica que se encuentra asociada a la membrana mitocondrial interna. Cada uno de los monómeros tiene un tamaño de 51 kDa y contiene un sitio de unión de dinucleótido de flavina adenina (FAD) cercano a su región N-terminal y un sitio de unión del sustrato [260, 261]. Se han detectado más de 100 mutaciones en el gen de la PPOX que conllevan la aparición de la enfermedad. La PV muestra una menor heterogeneidad alélica que las que presentan otras porfirias hepáticas. Es una enfermedad más común en Sudáfrica (con una prevalencia de 3:1000 habitantes [262]) y en Finlandia (con una prevalencia de 1,9:100000 habitantes) [263]. Hasta el momento no hay datos oficiales de prevalencia de PV en las Islas Baleares, pero la Asociación Balear de Porfiria estima que hay alrededor de 40 individuos afectados de PV y que han sido examinados exhaustivamente (incluyendo análisis bioquímicos y genéticos), pero también hay cerca de 100 individuos más que también podrían estar afectados y están pendientes de caracterización completa.

El cuadro clínico no se ve influenciado por el tipo de mutación en la forma heterozigota de la PV [264]. Se han descrito muy pocos casos de porfiria variegata homozigota. En estos casos, todos los pacientes mostraron fotosensitividad severa, tasa de crecimiento disminuida, anormalidades esqueléticas en las manos y niveles incrementados de protoporfirina en eritrocitos. Algunos de los pacientes mostraron síntomas neurológicos como retraso mental y convulsiones.

Las manifestaciones cutáneas son similares a las de la porfiria cutanea tarda: dermatitis fotoinducida causada por la acumulación de porfirinas en la piel, presentándose en forma de ampollas y erosiones en las zonas expuestas a la radiación solar. Los ataques agudos en la porfiria variegata son provocados por los mismos factores desencadenantes en otros tipos de porfiria (infecciones, ayuno, menstruación, estrés, alcohol y medicamentos) y cursan con dolor abdominal, vómitos, diarrea, hipertensión y taquicardia [265, 266]. La manifestación clínica de la enfermedad tiene lugar normalmente después de la pubertad, pero se especula que más del 50% de los portadores del gen mutado permanecen asintomáticos durante toda su vida [265].

Durante los ataques agudos de porfiria variegata, el resultado de los análisis de orina es muy similar al encontrado en PAI y CH [267]. Los portadores asintomáticos o que presentan sólo manifestaciones cutáneas crónicas a menudo muestran niveles ligeramente incrementados de ALA en orina [257]. La diferenciación de PV y CH es posible con análisis de porfirinas fecales. En la PV se encuentran niveles fecales elevados de protoporfirina y, en menor caso, coproporfirina. Otro de los marcadores diagnósticos característicos de la porfiria variegata es un pico de emisión máximo de fluorescencia en plasma a 624-628 nm [268].

La alimentación es importante en el tratamiento y prevención de las porfirias ya que el proporcionar una ingesta alta de hidratos de carbono y energía minimiza el número y gravedad de los ataques de porfiria aguda. Las recomendaciones dietéticas para la porfiria aguda son las siguientes: ingesta de energía que mantenga el peso corporal, ingesta de hidratos de carbono entre un 50-60% de la energía, ingesta de proteínas ajustada a la cantidad diaria recomendada para la población en general, ingesta total de grasa por debajo del 30% del total de energía, la ingesta de vitaminas y minerales ajustada a las RDA, la ingesta de fibra tiene que ser superior a 40g e inferior de 50g día. Sin embargo, no se conocen con certeza las necesidades de nutrientes antioxidantes y los efectos del consumo de altas dosis sobre el estado de estrés oxidativo y sus manifestaciones en este tipo de enfermos.

3.3.6. Porfiria y estrés oxidativo

Los niveles plasmáticos de ALA se encuentran incrementados cerca de 100 veces en los enfermos de PAI comparado con individuos sanos [269]. La oxidación aeróbica del ALA genera anión superóxido y peróxido de hidrógeno, y éstos a su vez pueden oxidar la oxihemoglobina a metahemoglobina [270], generar radicales hidroxilo (·OH) [270], inducir estrés oxidativo in vivo [271], inducir oxidación y rotura del DNA [272-274] y causar disfunciones en mitocondrias aislada [275]. En enfermos de PAI se describieron niveles elevados de SOD y glutatión peroxidasa en eritrocitos [276]. Estos estudios, tomados en su conjunto, sugieren que las porfirias hepáticas son enfermedades en las que el estrés oxidativo inducido por la acumulación de ALA juega un papel clave en el desarrollo patológico. Los niveles plasmáticos de ALA también se ven notablemente incrementados en las personas que han sido expuestas al plomo de forma prolongada y se ha observado una correlación entre los niveles plasmáticos de plomo y ALA, así como entre los niveles de ALA y de metahemoglobina [277].

El ALA se acumula en el cerebelo y la corteza cerebral produciendo alteraciones del metabolismo del hierro, daño en receptores GABA y estrés oxidativo. Los enzimas de síntesis de hemo han sido detectados en cerebro, lo que indica que el tejido nervioso tiene la capacidad de sintetizar ALA [278]. Estudios *in vitro* han demostrado que el ALA tiene efectos en toda una serie de funciones nerviosas, pero se ha descartado un papel farmacológico puesto que no es probable que el compuesto sea tóxico directamente en las relativamente bajas concentraciones presentes en el tejido nervioso durante un ataque porfírico agudo. Sin embargo, se ha demostrado que el ALA puede acumularse en la corteza cerebral [279, 280] y que las especies reactivas de oxígeno producidas por este compuesto pueden dañar los lípidos y las proteínas celulares, así como alterar el metabolismo del hierro [281-283].

El hecho de que el ALA forme radicales libres durante su autooxidación es la base para un modelo de la porfiria como un proceso dirigido por la generación de especies reactivas de oxígeno, y agravado por un incremento en el estrés oxidativo secundario a los incrementos temporales en la producción de ALA. [284]. Se ha demostrado incluso que el ALA puede producir daño oxidativo en los mismos enzimas de la ruta de síntesis del grupo hemo, potenciando así los efectos de la enfermedad [281]. También actúa sobre los niveles de hierro libre induciendo la liberación de hierro de la ferritina a través de modificaciones en las estructuras primaria, secundaria y terciaria de la proteína inducidas por oxidación [285], y aumentando la síntesis y los niveles de esta proteína [283, 286, 287]. La acumulación de grandes cantidades de ALA en enfermos de porfiria parece estar relacionada con la mayor incidencia de

cáncer en estos sujetos, que puede llegar a ser de hasta 70 veces mayor en el caso de cáncer de hígado [288].

Junto a los efectos derivados de la generación de especies reactivas de oxígeno por parte de los intermediarios metabólicos del grupo hemo, especialmente el ALA, la condición porfírica puede generar estrés oxidativo por otras vías. En enfermos de porfiria cutanea tarda se han detectado niveles plasmáticos disminuidos de vitaminas antioxidantes y niveles elevados de marcadores de daño oxidativo [289-291]. Debido a que el hemo es un constituyente esencial de un importante número de proteínas, las hemoproteínas, la dificultad a la hora de sintetizar el grupo prostético podría estar relacionada con un funcionamiento limitado de estas proteínas. Así, las limitaciones en la síntesis de grupo hemo podrían afectar a la funcionalidad de componentes de la cadena respiratoria mitocondrial como son los citocromos, induciendo una mayor producción de ROS, o a la funcionalidad de enzimas antioxidantes como la catalasa, mermando la capacidad detoxificadora de éstos.

4. Alimentación y estrés oxidativo

Numerosos estudios han puesto de manifiesto que el consumo de nutrientes antioxidantes proporciona una defensa efectiva contra los efectos perjudiciales de las ROS, y esto se traduce en un descenso en el riesgo de padecer ciertas enfermedades, especialmente enfermedades cardiovasculares y cáncer [292-294]. También se ha puesto de manifiesto el papel beneficioso de la ingesta adecuada de antioxidantes a la hora de prevenir las enfermedades asociadas al envejecimiento. Las personas mayores son una fracción de la población especialmente susceptible de padecer deficiencias en vitaminas y otros elementos esenciales. Recientemente se ha demostrado que los hábitos dietéticos que incluyen altas ingestas de antioxidantes pueden tener un efecto preventivo en la aparición de cáncer asociado a la edad [295, 296].

4.1. Alimentación y deporte. Suplementación con nutrientes antioxidantes

Como ha sido descrito anteriormente, la práctica de actividad física puede generar una situación de estrés oxidativo como consecuencia de la producción incrementada de especies reactivas de oxígeno. Para paliar estos efectos perjudiciales, una de las estrategias más recomendadas es la de diseñar una dieta rica en nutrientes antioxidantes que permitan que el deportista tenga en todo momento las defensas necesarias para neutralizar el estrés oxidativo. La planificación de una dieta

para el deportista se suele basar en evitar los contenidos dietéticos que pueden incrementar el riesgo de estrés oxidativo (dietas ricas en sal [297] y en grasas saturadas [298] y dietas hipercalóricas [299]) y en aumentar la ingesta de nutrientes con funciones antioxidantes. Una dieta rica en frutas y verduras eleva los niveles plasmáticos de vitaminas antioxidantes a la vez que reduce el riesgo de padecer enfermedades relacionadas con el estrés oxidativo [300]. Durante las pasadas décadas se han realizado un gran número de intervenciones con nutrientes antioxidantes en deportistas, con tal de alcanzar un consenso acerca de la conveniencia o inconveniencia de este tipo de suplementaciones. Sin embargo, este consenso no se ha llegado a alcanzar hasta el momento, debido a que los resultados obtenidos son muy variables. La razón de esta elevada variabilidad parece radicar tanto en los diferentes modelos de actividad física escogidos como generadores de estrés oxidativo, así como en la propia naturaleza de la intervención, ya sean el tipo y la dosis de los antioxidantes como la duración de la intervención.

La vitamina C ha sido ampliamente utilizada para contrarrestar los efectos adversos de la actividad física. Los primeros estudios sobre suplementación de deportistas con vitamina C se centraron en el rendimiento físico, pero sin encontrar beneficios al respecto [301, 302]. Otros resultados parecen sugerir que la suplementación puede incrementar el rendimiento físico únicamente cuando se parte de individuos con una deficiencia inicial de esta vitamina [303]. Una suplementación con vitamina C se ha descrito efectiva a la hora de prevenir la aparición de daño oxidativo [304] así como el dolor y daño muscular [305], y de disminuir la incidencia de resfriados en individuos físicamente activos [306]. La suplementación con vitamina C produce un aumento en los niveles basales plasmáticos de la vitamina [307] y un mayor aumento en los niveles plásmaticos tras un ejercicio intenso, además de incrementar la capacidad antioxidante total del plasma [308]. La vitamina C se encuentra en concentraciones elevadas en neutrófilos y es necesaria para su función en la respuesta inmune. La suplementación con vitamina C reduce la incidencia de infecciones del tracto respiratorio superior [309] y atenúa la respuesta inmunosupresiva posterior al ejercicio [310, 311]. Tras una suplementación con vitamina C, los neutrófilos muestran una respuesta diferencial al buceo en apnea, reduciendo la producción de NO, los niveles de iNOS y CAT, y la actividad de la glutatión peroxidasa [312]. También se ha propuesto que la vitamina C puede ayudar a disminuir el daño muscular producido por el ejercicio intenso y contribuir a la recuperación del músculo [313], posiblemente debido a la neutralización de los radicales libres liberados por los macrófagos reclutados en el tejido muscular dañado.

En el caso de la vitamina E los primeros estudios de suplementación también se centraron en sus efectos sobre el rendimiento y, al igual que con la vitamina C, no se encontraron beneficios al respecto [314-316]. Debido al papel protector de la vitamina E sobre la peroxidación lipídica de las membranas celulares, los estudios posteriores se han centrado en la habilidad de esta vitamina para disminuir el estrés oxidativo y el daño muscular. La suplementación con vitamina E durante tres meses provocó un incremento significativo de sus niveles plasmáticos, aumentando la protección de membranas y lipoproteínas frente a la peroxidación [317]. Además la vitamina E reduce la concentración de marcadores de peroxidación lipídica tales como el pentano expirado [169] o el MDA plasmático [318]. La vitamina E también parece proteger la integridad de la membrana de las células musculares ya que la suplementación reduce la liberación de enzimas musculares [319] y los niveles plasmáticos de interleuquinas inflamatorias [320]. La vitamina E también se ha mostrado efectiva en la reducción de daño en el DNA tras un ejercicio extenuante [321].

Hasta el momento se han realizado pocas intervenciones con coenzima Q en deportistas. La suplementación con coenzima Q se mostró efectiva a la hora de incrementar el rendimiento deportivo en varios experimentos [322-325], sin embargo otras intervenciones no evidenciaron efectos sobre el rendimiento deportivo [326-328], lo que sugiere que el coenzima Q puede ejercer una mejora modesta del rendimiento a pesar de que los resultados obtenidos no son del todo concluyentes. Un estudio con ratones evidenció que la suplementación de la dieta con coenzima Q no afecta a la actividad basal de los enzimas antioxidantes como la catalasa, glutatión peroxidasa y superóxido dismutasa en hígado, músculo esquelético o cerebro [329]. En el mismo estudio, la producción mitocondrial de especies reactivas, así como los niveles de grupos carbonilo y el ratio GSH:GSSG tampoco se vieron modificados por la suplementación con coenzima Q [329]. La administración de coenzima Q a humanos se ha comprobado efectiva a la hora de incrementar los niveles celulares del coenzima en linfocitos, y esta acumulación inhibe la aparición de daño oxidativo en el DNA y una potenciación de los enzimas de reparación de DNA [330]. En otro estudio se corroboró que la suplementación dietética con coenzima Q consigue aumentar sus niveles en linfocitos pero no así en leucocitos polimorfonucleares, a pesar de que en estos últimos dicha suplementación indujo un incremento en los niveles intracelulares de vitamina E [331]. Las propiedades antioxidantes del coenzima Q han llevado al estudio de los efectos de su administración en enfermedades relacionadas de forma directa o indirecta con el estrés oxidativo. En numerosos modelos animales de enfermedades

neurodegenerativas, tales como la esclerosis lateral amiotrófica, la enfermedad de Hungtington, o la enfermedad de Parkinson, la administración de CoQ_{10} ha mostrado tener efectos beneficiosos, aunque los estudios con humanos no son todavía concluyentes [332]. De forma similar, hay abundantes evidencias de que la administración de CoQ_{10} puede ser beneficiosa en el tratamiento de enfermedades cardiovasculares como la hipertensión o fallos cardíacos [333].

Junto con las suplementaciones individuales anteriores también se han realizado numerosos estudios en los que se ha suplementado a los deportistas con un combinado de antioxidantes. Se cree que la combinación de vitamina C y vitamina E es más efectiva que su suplementación individual debido a la capacidad de la vitamina C para regenerar la vitamina E. Si bien en un estudio la suplementación combinada de vitaminas E y C produjo una reducción significativa en los niveles de CK plasmáticos tras un ejercicio intenso [334], en otro estudio similar no se obtuvieron estas diferencias en cuanto a los niveles de CK y citoquinas [335]. Otros estudios de suplementación con ambas vitaminas determinaron un descenso en los niveles de pentano expirado y MDA sérico, así como en la velocidad de formación de lipoperóxidos [336, 337]. Una suplementación combinada de vitaminas E y C y β-caroteno aumentó la concentración plasmática de todos sus componentes y mostró una mayor sensibilidad de los linfocitos respecto de los eritrocitos a la suplementación, mejorando la respuesta de los enzimas antioxidantes al entrenamiento y al ejercicio agudo [338]. Una suplementación con vitamina C y glutatión evitó el incremento de GSSG en sangre tras un ejercicio hasta el agotamiento observado antes de la suplementación [339]. También se han realizado estudios de suplementación con nutrientes antioxidantes en buceadores con escafandra. Un estudio comprobó que una suplementación con vitaminas E y C y catequinas del té era capaz de prevenir las alteraciones hepáticas provocadas por el descenso a elevadas profundidades [340].

A pesar de las propiedades antioxidantes beneficiosas anteriormente mencionadas, en la actualidad se mantiene abierto un debate acerca de la posibilidad de que la suplementación con antioxidantes interfiera en el papel regulador de las ROS y en la respuesta adaptativa del propio organismo frente al estrés oxidativo. Se ha descrito la existencia de correlaciones directas entre el daño oxidativo en células sanguíneas y la actividad de varios enzimas antioxidantes, lo que sugiere que esta activación está mediada por ROS o por los productos derivados de la oxidación de biomoléculas [240]. Así mismo, se ha comprobado que la actividad física provoca un incremento en la actividad de enzimas antioxidantes como la catalasa y la SOD y la expresión de proteínas de choque térmico (HSP) en linfocitos, y que la suplementación

con vitamina C (500 mg diarios durante 8 semanas) evita estos incrementos tras el ejercicio, si bien a nivel basal los individuos suplementados presentaban unas mayores actividad enzimática y contenido en HSP [341]. Por tanto es de vital importancia que se establezca una ingesta óptima de nutrientes antioxidantes que evite la deficiencia en el nutriente pero también su toxicidad o interferencia en las mencionadas vías de adaptación. Para ello resulta fundamental el determinar las necesidades específicas de antioxidantes asociadas a cada tipo de deporte, y más allá incluso las necesidades individuales de cada deportista. Mientras continua la controversia sobre si los deportistas deben tomar suplementos de antioxidantes en concentraciones que superan ampliamente las recomendaciones diarias recomendadas, en general se recomienda la ingesta de una dieta rica en antioxidantes.

4.2. Alimentación y porfiria. Suplementación con nutrientes antioxidantes

La alimentación es un factor importante en el tratamiento y prevención de las porfirias. Por regla general, los afectados de porfiria no necesitan seguir una dieta especial, y las recomendaciones son aproximadamente las mismas que para la población general. Sin embargo, se han formulado algunas recomendaciones dietéticas específicas para ciertos tipos de porfiria En las porfirias agudas se recomienda una ingesta elevada de carbohidratos, ya que se ha demostrado que los ataques agudos de porfiria se pueden potenciar por ingestas restringidas de carbohidratos y energía. Por tanto, la ingesta de niveles normales o incluso ligeramente incrementados de carbohidratos y energía puede ayudar a prevenir las crisis porfíricas [247]. En el caso de porfirias no agudas (como la porfiria cutanea tarda o la porfiria eritropoyética) el consumo elevado de carbohidratos no parece importante. De hecho una de las recomendaciones dietéticas para afectados de porfiria cutanea (PCT) tarda ha sido una dieta de muy baja energía (VLED) y rica en fibra de verduras y frutas. La aportación de este tipo de dieta a pacientes con PCT reestableció los niveles de hierro sérico y ferritina, inicialmente elevados, hasta valores normales junto con una disminución en la excreción de coproporfirinas [342]. Otros estudios establecen recomendaciones dietéticas con una ingesta de energía superior, que mantenga el peso corporal, en la que los hidratos de carbono aporten entre un 50 y un 60% de la energía, una ingesta de proteínas ajustada a la cantidad diaria recomendada para la población general, una ingesta de grasa por debajo del 30% del total de energía, una ingesta de fibra entre 40-50 g/día, y una ingesta de vitaminas y minerales ajustada a la cantidad diaria recomendada. Hasta el momento no está del

todo consensuada la cantidad de energía que deben ingerir los afectados de porfiria, pero las recomendaciones coinciden en aportar una dieta pobre en grasa y rica en fibra.

Debido a las evidencias de que la enfermedad de la porfiria puede conllevar una situación de estrés oxidativo y daño celular, parece evidente la necesidad de que estos individuos ingieran cantidades suficientes de antioxidantes con tal de minimizar los posibles efectos adversos asociados a la enfermedad. A pesar de no haberse llegado a establecer unas recomendaciones generales en cuanto a la ingesta de vitaminas y otros nutrientes antioxidantes, se han realizado diferentes estudios durante los últimos años tratando de esclarecer los efectos que pueda tener la suplementación con antioxidantes sobre los marcadores de la enfermedad y el estrés oxidativo. En 1986 se describieron en enfermos de porfiria aguda intermitente (PAI) los efectos beneficiosos de una terapia antioxidante compuesta de ácido fólico, jarabe de glucosa, vitamina B, pantotenato de calcio, vitamina C, nicotinamida y vitamina E [343]. Sin embargo, en otro estudio más reciente la suplementación con un cóctel de antioxidantes (Vitaminas E y C, b-caroteno, selenio, coenzima Q) durante ocho semanas en enfermos de PAI no produjo ninguna mejora de los síntomas de la enfermedad [344]. En afectados de PCT se ha descrito una disminución en la excreción de porfirinas en respuesta a un tratamiento con dosis elevadas (1 g/día durante 30 días) de vitamina E [345]. Un estudio en ratones a los que se les indujo la aparición de porfiria con hexaclorobenceno (compuesto utilizado habitualmente para inducir porfiria en modelos animales) mostró que los niveles incrementados de porfirinas hepáticas, peróxidos lipídicos y 8-OHdG eran atenuados tras la administración de vitamina E [346].

También se han probado terapias con antioxidantes no nutricionales, como pueden ser la melatonina y la bilirrubina. La melatonina resulta efectiva a la hora de prevenir la formación de MDA y de restaurar las actividades de la porfobilinógeno deaminasa (PBG-D) y la ALA deaminasa (ALA-D) [347] y de reducir los niveles de DNA nuclear y lípidos de membrana oxidados [348] tras la inyección de ALA en ratas. Por otro lado la bilirrubina también ha mostrado efectos protectores frente a la inducción de porfiria por inyección de ALA, reduciendo los niveles de peroxidación lipídica y la actividad ALA sintasa, e incrementando la actividad de los enzimas antioxidantes catalasa, glutatión peroxidasa y superóxido dismutasa, y de los enzimas del metabolismo del grupo hemo PBG-D y ALA-D [349].

Los resultados obtenidos hasta el momento indican que una terapia rica en antioxidantes parece ser positiva a la hora de controlar ataques agudos de ciertos tipos de porfiria. La elección de los antioxidantes adecuados, usados por separado o

en combinación con otros tratamientos más convencionales, pueden llevar a desarrollar una nueva aproximación terapéutica para los enfermos de porfiria.

5. Efectos del género sobre el estrés oxidativo

Numerosos estudios durante los últimos años han puesto de manifiesto la existencia de diferencias de género en cuanto a las defensas antioxidantes y la aparición de estrés oxidativo. En general, las hembras parecen estar más protegidas que los machos frente al estrés oxidativo, tanto basal como el acontecido después de realizar actividad física intensa. Se ha visto que las hembras poseen mayores cantidades de glutatión reducido en sangre y mayores actividades glutatión peroxidasa y superóxido dismutasa en eritrocitos [350]. Estas mayores defensas antioxidantes se ven acompañadas por la presencia de un descenso en los niveles de marcadores de estrés oxidativo tales como MDA y sustancias reactivas del ácido tiobarbitúrico (TBARS) en plasma como marcadores de peroxidación lipídica [351] y un descenso en los niveles urinarios de 8-hidroxideoxiguanosina como marcador de oxidación en el DNA [352]. Se ha postulado que algunas de estas diferencias pueden ser atribuidas a las propiedades antioxidantes de las hormonas sexuales femeninas [353, 354]. De hecho, se ha comprobado que los estrógenos se unen al receptor de estrógenos e incrementan la expresión de enzimas antioxidantes tales como superóxido dismutasa y glutatión peroxidasa [355]. Estudios experimentales y clínicos sugieren que la expresión y la actividad de la superóxido dismutasa (SOD) son mayores en hembras que en machos [356-358] y que éstas están reguladas por los niveles de progesterona y estrógenos [358], lo que sugiere la existencia de complejas interacciones entre ambas hormonas a la hora de controlar la actividad SOD. La expresión de glutatión reductasa (GRd) se ve incrementada bajo concentraciones micromolares de estrógenos en células endoteliales, mientras que la progesterona y la testosterona no ejercen ningún efecto sobre dicho enzima [359]. En cuanto a la glutatión peroxidasa (GPx), algunos autores han descrito que su expresión y actividad son mayores en ratas hembras que en machos [356]. Sin embargo en leucocitos polimorfonucleares humanos no se detectaron diferencias de género [357]. Esta mayor protección frente al estrés oxidativo podría estar relacionada con la mayor esperanza de vida de las hembras comparadas con los machos [360].

También se han descrito un dimorfismo sexual en la modulación inmune. Los estrógenos y andrógenos parecen ser los responsables de estas diferencias en la producción de citoquinas inflamatorias. La testosterona puede suprimir la expresión de las citoquinas proinflamatorias TNF-α, IL-1β e IL-6, y potenciar la expresión de la

citoquina antiinflamatoria IL-10 [361]. Sin embargo, otros autores han descrito que la testosterona inhibe la activación de la óxido nítrico sintasa (NOS) y la fosforilación de PKB/Akt, provocando una mayor respuesta inflamatoria [362]. También se han detectado interacciones complejas entre los estrógenos y el TNF-α, que incrementa la producción de ROS e inhibe la eNOS. En un estudio mujeres menopáusicas presentaron mayores niveles séricos de TNF-α en comparación con mujeres premenopáusicas [363], y posteriormente se comprobó que los niveles circulantes incrementados de TNF-α estaban relacionados con una disminución de la expresión de NOS endotelial y un incremento en la expresión de NADPH oxidasa en arterias mesentéricas [364]. Numerosos estudios indican que tanto la nNOS como la eNOS están reguladas positivamente por estrógenos [365]. Niveles fisiológicos de estrógenos modulan directamente la expresión de nNOS, principalmente en neutrófilos. Así, se han documentado cambios en los niveles circulantes de productos de oxidación del $NO^{\cdot}$ durante el ciclo menstrual, así como durante una terapia de sustitución de estrógenos [366]. También se han detectado diferencias de género en la actividad de la mieloperoxidasa (MPO), enzima generador de especies reactivas presente en neutrófilos. Los hombres presentan por lo general recuentos de neutrófilos inferiores a las mujeres, probablemente debido a diferencias en la supervivencia del neutrófilo, que es mayor en mujeres [367]. El estradiol, a concentraciones fisiológicas, afecta a la función del neutrófilo potenciando la degranulación de neutrófilos activados, incluyendo la MPO, lo que podría llevar a un incremento de los productos prooxidantes [368].

Las hormonas sexuales también juegan un papel muy importante en la mitocondria. La testosterona, el 17β-estradiol y la progesterona modulan la expresión de factores nucleares (PPARγ, PGC1α, TFAM, etc.) implicados en el control de la biogénesis mitocondrial y la función termogénica. El 17β-estradiol activa la vía Akt/proteína quinasa B, mientras que la progesterona estimula la mitocondriogénesis y la diferenciación celular incrementando la expresión de factores nucleares como el TFAM. La testosterona, por su parte, reduce la transcripción del PGC1α, uno de los factores principales de la biogénesis mitocondrial [369]. La exposición a estrógenos produce una inmediata generación mitocondrial de ROS. Este incremento de las ROS activa la unión de tres factores de transcripción (AP-1, CREB y NRF1), actuando así como unos transductores de señal en la vía de los estrógenos [370]. Recientemente también se han detectado diferencias de género en cuanto a la susceptibilidad apoptótica relacionadas con diferencias en la producción de ROS [371].

Actualmente se está estudiando el posible papel de las diferencias de género asociadas a la producción y eliminación de especies reactivas en la patofisiología humana. Se ha comprobado que las actividades de las ROS están reguladas de forma diferencial en hombres y mujeres [365]. Por tanto, las diferencias en el estado redox asociadas al género podrían explicar, al menos parcialmente, las diferencias observadas entre los dos géneros en el mantenimiento de la homeóstasis del sistema inmune. El análisis de estas diferencias podría ser de gran relevancia a la hora de desarrollar nuevas estrategias terapéutica y en el desarrollo de una medicina del género [372].

II. OBJETIVOS Y PLANTEAMIENTO EXPERIMENTAL

1. Objetivo

Numerosas situaciones fisiológicas y patológicas están relacionadas con una incrementada producción de especies reactivas de oxígeno. La respuesta de las células a estos mayores niveles de especies reactivas difiere en función de la persistencia del estímulo. La práctica de episodios aislados de actividad física de alta intensidad supone incrementos puntuales en los niveles de especies reactivas e induce toda una serie de cambios y adaptaciones en las células del organismo que han sido atribuidas principalmente a estos incrementos en la producción de especies reactivas de oxígeno. Cuando la actividad física no se realiza de forma aislada, sino de forma regular y continuada (durante un periodo de entrenamiento), los estímulos a los que hace frente la célula son diferentes a los anteriores y por tanto su respuesta también diferirá. De la misma forma, determinadas enfermedades que conllevan una mayor producción de especies reactivas someten al organismo a condiciones hiperoxidativas persistentes a las que éste debe hacer frente de manera continua. Durante los últimos años se han hecho importantes avances en el estudio del papel regulador de las especies reactivas, y se ha puesto de manifiesto que la respuesta antioxidante está, al menos parcialmente, orquestada por las propias especies reactivas de oxígeno. Sin embargo, a pesar de este papel regulador, si los niveles o la persistencia de estas especies reactivas son lo suficientemente elevados, la célula puede verse sometida a un estado pro-oxidativo en el cual las biomoléculas celulares presentan una mayor susceptibilidad a la oxidación y a la pérdida de su función. Resulta pues evidente la necesidad de profundizar en el fino (des)equilibrio oxidativo que se establece ante incrementos relativamente modestos en los niveles de especies reactivas y determinar sus efectos sobre la funcionalidad celular. Los suplementos nutricionales con antioxidantes han sido ampliamente utilizados para aumentar las defensas antioxidantes del organismo frente a situaciones de riesgo oxidativo. Si bien clásicamente se optó por el uso de altas dosis de antioxidantes para obtener una mayor protección, el descubrimiento de la importancia de las propias especies reactivas en la respuesta adaptativa endógena al estrés oxidativo supuso un cambio de estrategia a la hora de diseñar estos suplementos antioxidantes, puesto que la utilización de altas dosis de estos compuestos podía repercutir de forma negativa en la adaptación celular. A pesar de que durante muchos años se ha probado una gran diversidad de suplementaciones antioxidantes, la variabilidad de los resultados hace difícil generalizar unas conclusiones definitivas. Existe controversia sobre el papel que tienen los antioxidantes en la regulación del desequilibrio oxidativo y en la prevención de patologías de gran impacto social. Conceptos como el de hormesis se están

aplicando a las especies reactivas de oxígeno, de manera que aquellas situaciones que potencian una cierta producción de especies reactivas de oxígeno se manifiestan como beneficiosas para la salud, proporcionando una adaptación que evita los posibles daños de una exposición excesiva a las especies reactivas de oxígeno. Este concepto de hormesis se ha aplicado al ejercicio moderado y también a los efectos que puede producir una restricción calórica en el envejecimiento. Así pues, no son tanto las especies reactivas las responsables en sí de producir efectos beneficiosos o negativos como son sus niveles y las tasas de su producción las responsables de que los efectos sean unos u otros. En este contexto, el consumo de antioxidantes en la dieta adquiere una nueva dimensión ya que se precisan criterios que permitan el establecimiento de las ingestas que optimicen sus efectos beneficiosos y del diseño de alimentos con niveles adecuados para conseguirlas. De esta forma, se continúa en la búsqueda de un suplemento nutricional, o un alimento funcional, que aporte una dosis efectiva de antioxidantes para hacer frente al estrés oxidativo sin que inhiba la respuesta adaptativa celular, y más aún que potencie las defensas antioxidantes endógenas del organismo. Es preciso evidenciar el mecanismo que subyace en los posibles efectos de las especies reactivas de oxígeno y de los antioxidantes sobre las defensas antioxidantes endógenas y el daño oxidativo. Por ello es necesario disponer de situaciones fisiológicas en las que el desequilibrio oxidativo pueda inducirse o bien tenga un carácter crónico y evaluar la evolución que el consumo del suplemento antioxidante induce sobre el daño oxidativo y la respuesta antioxidante adaptativa.

El objetivo general de esta tesis es encontrar situaciones y estrategias que potencien las defensas antioxidantes endógenas, aportando una mayor protección frente a la aparición de estrés oxidativo, y profundizar en los mecanismos implicados en esta adaptación celular.

El objetivo general se divide a su vez en los siguientes objetivos específicos:

1.- Determinar la respuesta de los sistemas antioxidantes en células sanguíneas y plasma, así como la posible inducción de estrés oxidativo, asociada a la hiperoxia e hiperbaria.
2.- Determinar la respuesta de los sistemas antioxidantes en células inmunitarias y plasma, y su repercusión en el daño oxidativo, a diferentes tipos de ejercicio físico.
3.- Establecer mecanismos de respuesta antioxidante endógena de las células inmunitarias frente al estrés oxidativo.

4.- Identificar adaptaciones endógenas diferenciales al estrés oxidativo en función del género.

5.- Caracterizar la porfiria variegata como un modelo humano de estrés oxidativo crónico asociado a una patología oxidativa.

6.- Evidenciar efectos potenciadores de nutrientes antioxidantes sobre las defensas antioxidantes endógenas de las células sanguíneas y plasma.

7.- Desarrollar un modelo de cultivos celulares en el que mimetizar el efecto dual de las especies reactivas de oxígeno como mensajeros e inductoras de daño oxidativo.

2. Planteamiento experimental

Para alcanzar los objetivos propuestos en la presente tesis se planificaron toda una serie de experimentos utilizando diferentes modelos experimentales de generación de estrés oxidativo. En todos los experimentos en los que participaron humanos se cumplió con los requisitos indicados en la Declaración de Helsinki, y todos los protocolos fueron previamente aprobados por la Comisión de Ética del Hospital Universitario de Son Dureta de Palma de Mallorca o la Comisión de Ética de Investigación Clínica de la CAIB. Todos los participantes accedieron voluntariamente a su participación tras haber sido debidamente informados del objetivo del estudio y de los posibles riesgos.

A continuación se describe el planteamiento experimental desarrollado, el cual se ha estructurado parcialmente en función de los objetivos concretos de la tesis.

2.1. Estrés oxidativo asociado a la hiperbaria y respuesta de las defensas antioxidantes

Para alcanzar el objetivo 1 desarrollamos un diseño experimental fundamentado en la realización de una inmersión con escafandra a 40 metros de profundidad en la que se produce una situación de hiperbaria y a la vez se realiza una actividad física. Siete buceadores pre-profesionales realizaron una inmersión a 40 metros durante 25 minutos en la que respiraron una mezcla de aire atmosférico. El esquema de la sesión de buceo se muestra en la Figura 5. Los buceadores tardaron 3 minutos en alcanzar la profundidad de 40 metros, donde permanecieron 10 minutos. Transcurrido dicho tiempo realizaron la ascensión durante 5 minutos y una descompresión de 5 minutos a una profundidad de 3 metros. Se recogieron muestras

de sangre venosa antes e inmediatamente después de la inmersión y tras 3 horas de recuperación.

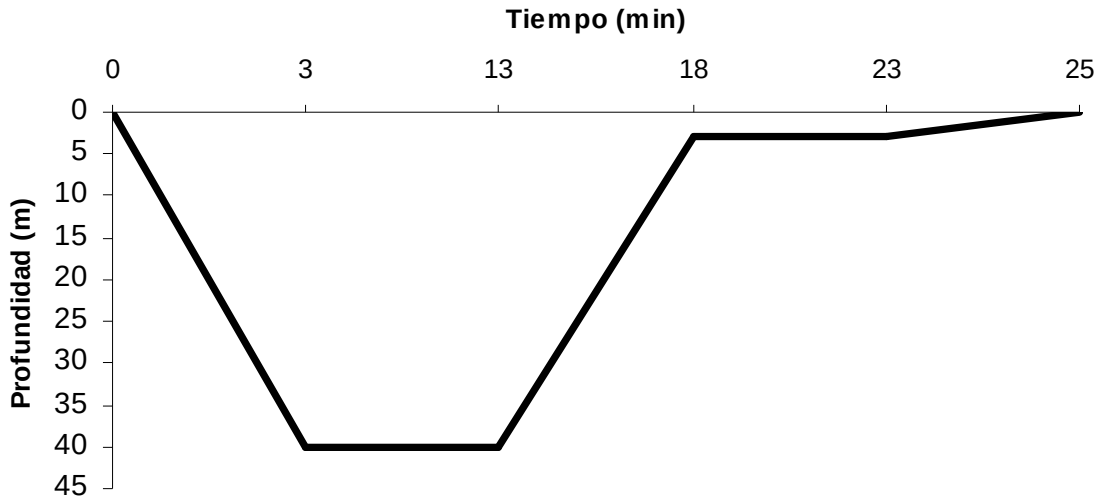

Figura 5. Perfil de la inmersión en la sesión de buceo estudiada.

Con el fin de identificar si la hiperbaria por sí sola es capaz de inducir los cambios asociados al buceo con botella, se desarrolló un segundo estudio en el que se realizó una exposición seca a oxígeno hiperbárico en una cámara hiperbárica. 12 sujetos físicamente activos respiraron oxígeno al 100% a una presión de 2,2 ATA durante 60 minutos en una cámara hiperbárica. Se recogieron muestras de sangre venosa antes y 30 minutos después de finalizar la exposición hiperbárica.

En linfocitos se determinó la actividad de los enzimas antioxidantes catalasa y glutatión peroxidasa y de la caspasa-3, los niveles de nitrito e iNOS, la producción de H_2O_2 y la expresión del gen de la hemooxigenasa 1 (HO-1). En neutrófilos se determinó la actividad mieloperoxidasa (MPO) y la producción de especies reactivas de oxígeno por quimioluminiscencia. Como marcadores de daño celular se determinaron las actividades creatina quinasa (CK) y lactato deshidrogenasa (LDH). En plasma y eritrocitos se determinaron las actividades de enzimas antioxidantes, los niveles de nitrito y los niveles de MDA e índice de carbonilos como marcadores de oxidación de lípidos y proteínas, respectivamente.

2.2. Respuesta antioxidante y aparición de daño oxidativo frente al ejercicio físico. Mecanismos de respuesta antioxidante.

Estudiamos el efecto de diferentes tipos de actividad física sobre los sistemas antioxidantes, así como la influencia de la intensidad del ejercicio y el entrenamiento. Para tratar de alcanzar los objetivos 2 y 3 desarrollamos tres planteamientos experimentales diferentes. En el primero de ellos trabajamos con un equipo de fútbol y determinamos su nivel antioxidante en diferentes puntos de la temporada de competición y analizamos los efectos que tiene un partido de fútbol sobre las defensas antioxidantes y el daño oxidativo. En el estudio participaron 19 jugadores de las categorías inferiores del F. C. Barcelona. El inicio del estudio coincidió con el inicio de la temporada en invierno, momento en el que los futbolistas se encuentran en un menor estado de entrenamiento. Se tomaron muestras de sangre en condiciones basales y tras la realización de un partido de entrenamiento de 60 minutos. La intensidad del ejercicio realizado durante este partido por cada jugador se determinó utilizando las medidas registradas en un pulsímetro. Los futbolistas se dividieron en tres grupos en función de la intensidad del ejercicio realizado durante el partido: Baja intensidad (más del 50% de la duración del partido trabajaron por debajo del 80% del VO_2max), media intensidad (más del 50% del partido trabajaron entre el 80 y el 90% del VO_2max), y alta intensidad (más del 50% del partido trabajaron a un nivel superior al 90% del VO_2max). Se tomaron muestras de sangre antes e inmediatamente después del partido de fútbol y se determinaron el recuento de neutrófilos y la producción de ROS por parte de éstos. En los linfocitos se determinaron los niveles de MDA y vitaminas C y E, así como la expresión del gen hemooxigenasa 1 y la producción de ROS.

Para estudiar los efectos del entrenamiento sobre los sistemas antioxidantes tomamos nuevas muestras de sangre en condiciones basales en los mismos sujetos después de tres meses, coincidiendo con la recta final de la competición. En linfocitos y neutrófilos se determinaron las actividades de enzimas antioxidantes, los niveles de MDA y vitaminas antioxidantes, y la producción de ROS. Los niveles de vitaminas antioxidantes y MDA también se determinaron en plasma. Los resultados obtenidos en condiciones de reposo al inicio del período de entrenamiento y al final se compararon para determinar los efectos del entrenamiento. Tras este período de tres meses de entrenamiento los sujetos volvieron a jugar otro partido de entrenamiento de 60 minutos de duración, en el que todos estuvieron trabajando más del 50% del partido a un nivel superior al 80% de su respectiva VO_2max. Se recogieron muestras de sangre

antes e inmediatamente después del partido. Los parámetros determinados fueron los mencionados anteriormente en linfocitos, neutrófilo y plasma.

En un segundo experimento se utilizó una sesión intensa de natación como modelo inductor de estrés oxidativo asociado al ejercicio. En dicha sesión participaron 15 chicos y 9 chicas bien entrenados, pertenecientes a equipos de natación amateur. Todos los participantes efectuaron una sesión de calentamiento de 30 minutos antes de comenzar el protocolo de ejercicio. Éste consistió en series de 50 m a una velocidad incrementada progresivamente durante 30 minutos, descansando 10-15 segundos entre serie y serie, y llegando al cabo de los 30 minutos a alcanzar un ritmo correspondiente al 80% de la capacidad máxima individual, controlado por el tiempo que tardaban en completar los 50 m en comparación con su mejor tiempo obtenido en pruebas previas. Una vez alcanzado este ritmo, los participantes siguieron nadando durante 30 minutos manteniendo la intensidad alrededor del 75-80% de su capacidad máxima, y al igual que en la primera parte descansando 10-15 s entre series. Se obtuvieron muestras de sangre antes del ejercicio y entre una y dos horas después de haber finalizado la prueba. En neutrófilos y linfocitos se determinaron las actividades enzimáticas antioxidantes, los niveles de vitaminas C y E y los niveles de marcadores de oxidación en lípidos y proteínas. En linfocitos se determinó además la expresión de los genes catalasa, Mn-SOD, iNOS, sirtuina 3, UCP-3, Bcl-2 y PGC-1α, así como los niveles de nitrito y la producción de ROS. Para profundizar en las relaciones existentes entre todos estos parámetros se realizaron una serie de estudios estadísticos de correlación. En plasma se determinaron los niveles de vitaminas antioxidantes y de marcadores de daño oxidativo. En suero se determinaron diferentes parámetros séricos tales como las actividades lactato deshidrogenasa y creatina quinasa, y los niveles de ácido úrico, colesterol y triglicéridos.

En estudios anteriores a la presente tesis se había puesto de manifiesto que el neutrófilo responde al ejercicio físico intenso disminuyendo la actividad de sus enzimas antioxidantes. Esta disminución de la actividad catalasa ha sido atribuida a su posible salida del neutrófilo. Para profundizar en el mecanismo de regulación que subyace en esta respuesta del neutrófilo se diseñó un tercer experimento en el que se estudiaron dos etapas ciclistas llanas como modelo de ejercicio físico intenso que induce estrés oxidativo. Siete ciclistas profesionales participaron en este estudio y tardaron una media de 244 ± 11 minutos en completar cada etapa. Se tomaron muestras basales en la mañana anterior a cada etapa y 3 horas después de haberla finalizado. Se determinaron las actividades y expresión de los enzimas catalasa, superóxido dismutasa, glutatión peroxidasa y mieloperoxidasa, los niveles de MDA y grupos carbonilo en proteínas y la producción de ROS por parte del neutrófilo. La localización

celular de la catalasa antes y después de las etapas ciclistas se estudió por inmunocitoquímica. Para profundizar en la posible liberación de enzima catalasa al medio extracelular por parte del neutrófilo activado, se realizó un estudio *in vitro* adicional. Para ello se obtuvo una población de neutrófilos de un individuo en condiciones de reposo y se incubaron estos neutrófilos en presencia de zymosan opsonizado, un conocido activador de los neutrófilos. Las actividades catalasa y mieloperoxidasa se determinaron en el medio de cultivo antes y después de la activación con zymosan.

2.3. Diferencias de género en las adaptaciones endógenas al estrés oxidativo

Para estudiar las diferencias de género en las adaptaciones endógenas al estrés oxidativo utilizamos el modelo experimental anteriormente mencionado de la práctica de natación intensa durante una hora para inducir estrés oxidativo y respuesta endógena. En dicha prueba participaron 15 chicos y 9 chicas, por lo que aplicamos un tratamiento estadístico considerando el género como factor estadístico, y analizamos las diferencias encontradas atribuibles al género.

2.4. Estrés oxidativo asociado a la porfiria variegata

Para la consecución del objetivo 5 se ha diseñado un experimento en el que participaron mujeres afectadas de porfiria variegata y mujeres control, apareadas por edad y condición de fertilidad. La dificultad a la hora de sintetizar el grupo hemo que padecen los afectados por la enfermedad de la porfiria podría inducir a una mayor producción de especies reactivas de oxígeno a la vez que comprometer los mecanismos de defensa. En el estudio diseñado para determinar los efectos crónicos de la condición porfírica sobre las defensas antioxidantes y el estrés oxidativo participaron 12 mujeres afectadas de porfiria variegata y 12 mujeres control, sin ninguna patología reconocida y con edades ajustadas a las mujeres afectadas de porfiria. Todas las mujeres incluidas en el grupo de porfiria habían sido diagnosticadas previamente como portadoras de la enfermedad en base a parámetros diagnósticos tales como los niveles de porfirinas fecales y urinarias, presencia de un pico plasmático de emisión de fluorescencia a 626 nm, y manifestaciones clínicas como dolor abdominal, náuseas y vómitos durante los ataques porfíricos y síntomas cutáneos como erupciones y cambios de pigmentación.

De todos los sujetos participantes se tomaron muestras de sangre en condiciones basales (en reposo y tras un ayuno de toda la noche). Se determinaron

parámetros hematológicos tales como el recuento de eritrocitos y leucocitos, así como la fórmula leucocitaria, el hematocrito, el volumen corpuscular medio (VCM), la hemoglobina corpuscular media (HCM) y los niveles de hemoglobina. En eritrocitos se determinaron las actividades de los enzimas catalasa, glutatión reductasa, glutatión peroxidasa, superóxido dismutasa y ácido δ-aminolevulínico dehidratasa. También se determinaron en eritrocitos los niveles de proteína catalasa y protoporfirinógeno oxidasa, así como los niveles de MDA. En linfocitos se determinaron las actividades enzimáticas catalasa, glutatión peroxidasa, glutatión reductasa y superóxido dismutasa y los niveles de vitaminas C y E. El estudio de las defensas antioxidantes en el linfocito se extendió a la expresión de los genes catalasa, glutatión peroxidasa, hemooxigenasa 1, UCP-3, Bcl-2 y sirtuina 3. La capacidad de producción de ROS por parte del linfocito, así como los sistemas implicados en esta producción linfocitaria de ROS, también fueron estudiados. Para identificar las fuentes de ROS en el linfocito se utilizaron tres inhibidores diferentes: alopurinol (inhibidor de la xantina oxidasa), rotenona (inhibidor del complejo I de la cadena respiratoria) y mixotiazol (inhibidor del complejo III de la cadena respiratoria). Como marcadores de daño oxidativo en el linfocito se determinaron los niveles de MDA y grupos carbonilo y se realizó un estudio del estado del DNA mediante la técnica del *comet assay*.

2.5. Efectos de la suplementación de la dieta con antioxidantes

Una vez evidenciados y caracterizados los efectos que tienen las diferentes situaciones sobre la inducción de estrés oxidativo y alguno de los mecanismos que subyacen en la respuesta adaptativa antioxidante, pasamos a desarrollar el objetivo 6 y estudiar la potencialidad de diferentes suplementos nutricionales de activar las defensas antioxidantes y contrarrestar el estrés oxidativo. En un primer estudio de suplementación el modelo utilizado como inductor de estrés oxidativo fue el ejercicio físico, tanto agudo como crónico. En este estudio participaron 19 futbolistas pre-profesionales voluntarios, que fueron suplementados en un diseño a doble ciego con un combinado multivitamínico y mineral especialmente rico en coenzima Q, cuya composición se muestra en la Tabla 3, o con un placebo. Todos los participantes tomaron el suplemento o placebo durante 90 días. Tras los 90 días de suplementación los participantes jugaron un partido de fútbol de entrenamiento de 60 minutos de duración, y se obtuvieron muestras de sangre venosa antes y después del partido. Para valorar el estado de las defensas antioxidantes en linfocitos y neutrófilos, se determinó la actividad de los enzimas antioxidantes y los niveles de ascorbato y α-tocoferol. En plasma se determinaron los niveles de ascorbato, α-tocoferol y coenzima

Q. Para determinar la capacidad prooxidante y el daño oxidativo se determinó la producción de H_2O_2 en linfocitos, los niveles de MDA en linfocitos, neutrófilos y plasma y el índice de carbonilos en neutrófilos y plasma.

En el segundo estudio de suplementación se utilizó la condición de porfiria como modelo generador de estrés oxidativo. Las participantes formaron parte de un estudio a doble ciego cruzado. Cada sujeto bebió durante seis meses 500 ml/día de una bebida realizada a base de almendra y naranja enriquecida con vitamina E (10 mg/100 ml) y vitamina C (30 mg/100 ml), o una bebida idéntica pero no enriquecida con las vitaminas antioxidantes. Tras el período inicial de seis meses de suplementación se dejaron transcurrir tres meses y a continuación se cruzaron los grupos y se realizó una nueva suplementación de las mismas características durante seis meses más. De esta forma, cada participante ingirió tanto la bebida placebo como la suplementada. Después de ambos períodos de suplementación se obtuvieron muestras de sangre en condiciones de reposo y se determinó la fórmula leucocitaria. En linfocitos se determinaron los niveles de vitaminas C y E, las actividades de los enzimas antioxidantes, la expresión de los genes glutatión peroxidasa, glutatión reductasa, Mn-SOD y Cu/Zn-SOD, la producción de ROS y los niveles de MDA e índice de carbonilos.

Tabla 3. Composición del cocktail multivitamínico y mineral suministrado a futbolistas

Ingrediente	Dosis	Cantidad diaria recomendada (%)[a]
Vitamina A	1000 µg	125
Vitamina C	60 mg	100
Vitamina E	10 mg	100
Niacina	18 mg	100
Ácido pantoténico	4,0 mg	80
Vitamina B12	3,0 µg	125
Vitamina B6	2,2 mg	147
Vitamina B2	1,6 mg	100
Vitamina B1	1,1 mg	92
Vitamina D	2,5 µg	50
Ácido fólico	180 µg	45
Biotina	30 µg	100
Magnesio	100 mg	25
Zinc	7,5 mg	50
Selenio	50 µg	71
Cobre	1000 µg	91[b]
Manganeso	2,5 mg	No establecida
Coenzima Q_{10}	100 mg	No establecida

[a] Calculadas en base a los valores de ingesta diaria recomendada (IDR) para la población española [373]

[b] Calculada en base a la ingesta de referencia de la población europea (PRI) [374]

En un tercer estudio de suplementación participaron 10 nadadoras pertenecientes a equipos amateur. A las participantes se les proporcionó de forma aleatoria y a doble ciego 500 ml diarios de la misma bebida de almendra rica en vitaminas C y E anteriormente descrita (10 mg/100 ml de vitamina E y 30 mg/100 ml de vitamina C) o una bebida de las mismas características pero además suplementada con 400 mg/100 ml de extracto de *Lippia citriodora*, que contiene aproximadamente un 10% de verbascósido y un 5-8% de otros fenilpropanoides. Las participantes bebieron la bebida durante 26 días, y tras este período de intervención nutricional participaron en una sesión de entrenamiento de natación intensa. La sesión consistió en 30 minutos de series de 50 metros a una intensidad constante del 75-80% de la capacidad máxima individual, y con descansos de 10-15 segundos entre series. Se obtuvieron muestras de sangre antes y 1 hora después de la sesión de natación. Se determinaron parámetros hematológicos (hemoglobina, hematocrito, MCV, MCH, MCHC, RDW) y la fórmula leucocitaria. En plasma se determinaron los niveles de progesterona, 17-β-estradiol, testosterona, testosterona libre y proteína de unión a hormonas sexuales. Las actividades catalasa, glutatión peroxidasa y superóxido dismutasa se determinaron en linfocitos y eritrocitos. Los niveles de MDA e índice de carbonilos, así como las roturas en el DNA se determinaron en linfocitos. La expresión de los genes catalasa, glutatión peroxidasa, Mn-SOD, Bcl-2, UCP3 y hemooxigenasa también se determinaron en linfocitos.

6. Modelo *in vitro* de generación de estrés oxidativo

Las especies reactivas de oxígeno, cuando se encuentran a niveles elevados, pueden oxidar las biomoléculas celulares e inducir así la aparición de estrés oxidativo. Sin embargo, cuando estas ROS se encuentran a niveles fisiológicos actúan como mensajeros celulares en la adaptación endógena de la célula al estrés oxidativo, así como en otros procesos de señalización y respuesta. El séptimo objetivo de esta tesis pretendía desarrollar un modelo de cultivos celulares que mimetizara este efecto dual de las ROS y tratar de encontrar un tratamiento con H_2O_2 que indujera la adaptación de las defensas antioxidantes sin llegar a producir daño oxidativo. La línea celular con la que trabajamos fue la HL60, línea derivada de leucocitos periféricos obtenidos por leucoforesis de una paciente con leucemia promielocítica aguda. Escogimos esta línea celular puesto que, aunque se trata de una línea indiferenciada, sus características se asemejan a las de los leucocitos, objeto de estudio a lo largo de esta tesis. Las células fueron mantenidas en cultivo en medio RPMI 1640 suplementado con suero de ternera fetal inactivado al 10%, antibióticos (penicilina 100 U/ml, estreptomicina 0,1 ng/ml) y L-

glutamina 2 mM, en atmósfera húmeda con un 5% de CO_2 a 37ºC. Las células fueron cultivadas en *flasks* de cultivo de 250 ml a una concentración inicial de 2 x 10^5 células/ml.

Para cumplir con el objetivo propuesto, desarrollamos dos modelos experimentales, en los que las células fueron expuestas al tratamiento bien con una dosis puntual, bien con una dosis sostenida de H_2O_2. En el primer modelo, las células HL60 fueron incubadas en presencia de una concentración inicial de H_2O_2 de 1, 10 y 100 μM. Tras la adición del H_2O_2, las células fueron mantenidas en cultivo durante 2 horas, a pesar de que a los 60 minutos ya se hubo consumido la práctica totalidad del H_2O_2 en el medio. Tras las 2 horas de incubación, las células fueron recogidas y lavadas 2 veces con PBS. En las muestras obtenidas determinamos la actividad de los enzimas antioxidantes catalasa, glutatión peroxidasa, glutatión reductasa y superóxido dismutasa, los niveles de grupos carbonilo, la producción intracelular de H_2O_2, y la expresión de los genes catalasa, glutatión peroxidasa, Mn-SOD, HO-1, UCP-3, Bcl-2 y PGC-1α. En el segundo modelo, las células fueron incubadas durante 1 hora en presencia de unos niveles sostenidos de H_2O_2 obtenidos a partir de la adición del enzima glucosa oxidasa y su sustrato, la glucosa, que permitían la producción continua de H_2O_2. La presencia de los sistemas detoxificadores de la célula asegura que los niveles de H_2O_2 se estabilicen y que no se vayan acumulando hasta alcanzar niveles tóxicos para la célula [375]. Las producciones constantes de H_2O_2 obtenidas de esta forma fueron 0,1, 1 y 10 nM/s. Tras la incubación de 1 hora en presencia de dichas producciones de H_2O_2, las células fueron lavadas y cultivadas en medio fresco sin H_2O_2 durante 1 hora más. Tras esta segunda incubación, las células fueron de nuevo recogidas y lavadas 2 veces con PBS. En las muestras obtenidas determinamos la viabilidad celular, la actividad de los enzimas antioxidantes catalasa, glutatión peroxidasa, glutatión reductasa y superóxido dismutasa, los niveles de grupos carbonilo, la producción intracelular de H_2O_2, los niveles de nitrito extracelular, el daño oxidativo en el DNA por *comet assay* y la expresión de los genes catalasa, HO-1, UCP-3, iNOS y PGC-1α.

III. RESULTADOS Y DISCUSIÓN

RESULTS AND DISCUSSION

Manuscript I

Scuba diving enhances endogenous antioxidant defenses in lymphocytes and neutrophils.

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Scuba diving enhances endogenous antioxidant defenses in lymphocytes and neutrophils

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Abstract
The aim was to study the effects of a scuba diving session on the lymphocyte antioxidant system, NO synthesis, the capability to produce reactive oxygen species and the antioxidant response in neutrophils. For that purpose seven male divers performed an immersion at a depth of 40 m for 25 min. The same parameters were measured after an hyperbaric oxygen (HBO) treatment at resting conditions in a hyperbaric chamber. Lymphocyte H_2O_2 production rose after diving and after HBO treatment. Glutathione peroxidase (GPx) and catalase activities increased after diving in lymphocytes, while after HBO exposure only increased GPx activity. Lymphocyte HO-1 mRNA expression increased after diving and after HBO exposure, while iNOS levels and nitrite levels significantly increased after diving. The hyperoxia associated to scuba diving leads to a condition of oxidative stress with increased lymphocyte H_2O_2 production, HO-1 expression, NO synthesis and antioxidant enzyme adaptations in order to avoid oxidative damage.

Keywords: *Oxidative stress, scuba diving, hyperbaric oxygen, nitric oxide, iNOS, lymphocytes*

Introduction

Scuba diving is characterized by hyperoxia resulting from hyperbaric exposure during diving and the availability of oxygen at high pressure and could induce oxidative stress. In addition to this, diving also implies physical activity which, in itself, increases the production of reactive oxygen species (ROS). Hyperbaric oxygen (HBO) therapy provides 100% inhaled oxygen at increased atmospheric pressure. Exposure to HBO leads to a rise in the oxygen dissolved in blood, and has been successfully used as an adjuvant therapy for many disorders such as decompression sickness, acute carbon monoxide intoxication or impaired wound healing [1,2]. However, HBO has also been evidenced to lead to increased formation of ROS [3]. One single HBO exposure can induce oxidative stress, resulting in cellular damage with lipid peroxidation and protein and DNA oxidation [4–6].

The cellular response to HBO-induced oxidative stress has been mainly investigated in animal models [7,8] and only few studies have been performed in humans [9,10]. Enzymatic antioxidants of lymphocytes have demonstrated great adaptation to oxidative stress (e.g. exercise-induced) by increasing their activities [11,12]. Lymphocytes from healthy volunteers show a markedly increased HO-1 protein concentration after HBO exposure and induce adaptive protection against oxidative damage after a second HBO exposure [13].

Lymphocytes present the inducible isoform of NOS (iNOS) whose expression is induced by some cytokines such as IFN-γ, TNF-α and IL-1 [14]. In the lymphocyte NO also inhibits proliferation by arresting cell cycle progression at the G1 phase [14]. We previously evidenced a direct correlation between iNOS expression and SOD activity in the neutrophil and lymphocyte, which links NO synthesis to oxidative

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stress [15]. The administration of NO donors induces HO-1 expression, which plays an important role in the adaptive response to HBO-induced damage [16].

We hypothesize that HBO increases lymphocyte capabilities to produce ROS and induces the expression of antioxidant defenses in order to maintain the redox balance and to avoid oxidative damage in lymphocytes. Our aim was to study the effects of an HBO exposure at rest and a single scuba dive to a great depth on the lymphocyte antioxidant system, on lymphocyte NO synthesis and on their capability to produce ROS. We also determined the oxidative capability of the neutrophil, as the major source of ROS.

Materials and methods

Subjects and study design

Two different studies were performed. The first study consisted of a scuba diving session at a depth of 40 m. Seven male divers, aged: (26.0 ± 4.7 years), with body mass index, BMI: (23.1 ± 0.6 Kg/m^2) volunteered to take part in this study. The subjects were all non-smoker scuba diving learners and they did not take any antioxidant dietary supplement or any routine medication for one month prior to the study. Divers performed an immersion at a depth of 40 m for a total time of 25 min in which they breathed atmospheric air. The schedule of the scuba diving session is shown in Figure 1. Divers spent 10 min at 40 m, and the return to the surface was with a decompression of 5 min at a depth of 3 m. The pulses of the divers were measured using a Polar Electro S18 pulsometer, and the results were analyzed with Polar Precision Performance software version 3.

In order to identify if HBO alone is enough to induce scuba diving-associated changes we designed a second study consisting of a dry HBO exposure in a hyperbaric chamber. About 12 male physically active subjects, aged: (25.3 ± 3.9 years), with body mass index, BMI: (20.7 ± 2.3 Kg/m^2) were exposed at rest to a HBO treatment. The subjects were all non-smokers and they did not take any antioxidant dietary supplement or any routine medication for one month prior to the study. Subjects were exposed to 100% oxygen at a pressure of 2.2 ATA in a hyperbaric chamber for 60 min.

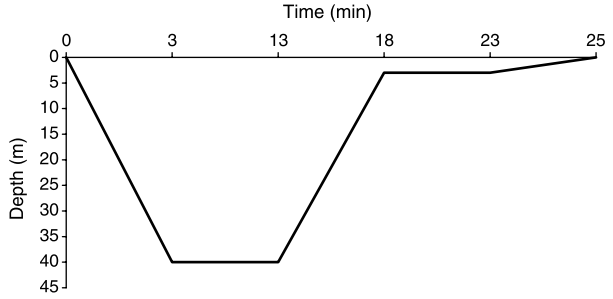

Figure 1. Schedule of the scuba diving session profile.

For both experiments the protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the CAIB (Palma de Mallorca, Balearic Islands, Spain). All the subjects were informed of the purpose and demands of the study before giving their written consent to participate.

Experimental procedure

Venous blood samples were obtained from the antecubital vein of divers and HBO patients in suitable vacutainers. In the scuba diving study, venous blood samples were obtained before the diving session after overnight fasting (basal sample), immediately after diving, and 3 h after finishing the diving session. In the HBO study, samples were obtained before the HBO exposure (after overnight fasting) and 30 min after finishing the treatment.

The lymphocyte and neutrophil fractions were purified. Antioxidant enzyme activities, caspase-3 activity, nitrite and iNOS levels were measured in lymphocytes. Heme oxygenase-1 (HO-1) mRNA expression and the production of hydrogen peroxide were also determined in lymphocytes. Myeloperoxidase (MPO) activity and the oxidative capacity were determined in neutrophils. Creatine kinase (CPK) and lactate dehydrogenase (LDH) were measured in serum using commercial clinical kits in an autoanalyser system (Technicon DAX System).

A whole blood aliquot was analyzed in an automatic flow cytometer analyzer Technicon H2 (Bayer) VCS system to determine lymphocyte and neutrophil counts.

Neutrophil and lymphocyte purification

Blood samples were processed following an adaptation of the method described by Boyum [17]. Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and was then centrifuged at 900*g*, 18°C for 30 min. The lymphocyte layer was carefully removed. The precipitate containing the erythrocytes and neutrophils were incubated at 4°C with 0.15 M ammonium chloride to hemolyse the erythrocytes. The suspension was centrifuged at 750*g*, 4°C for 15 min and the supernatant was then discarded. The neutrophil phase at the bottom was washed first with ammonium chloride and then with phosphate saline buffer, pH 7.4. Finally, the neutrophils were lysed with distilled water.

The lymphocyte slurry was then washed twice with PBS and centrifuged for 10 min at 1000*g*, 4°C. The cellular precipitate of lymphocytes was lysed with distilled water.

Antioxidant enzyme and MPO activities

Catalase (CAT) activity was measured by the spectrophotometric method of Aebi based on the decomposition

of H_2O_2 [18]. Glutathione peroxidase (GP) activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler [19]. Neutrophil MPO activity was measured by guaiacol oxidation [20]. The reaction mixture contained sodium phosphate buffer pH 7 and 13.5 mM guaiacol. The reaction was initiated by adding 300 μM H_2O_2, and changes were monitored at 470 nm. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37°C.

Caspase-3 activity

Caspase-3 activity was measured in lymphocytes by using a spectrophotometric assay using the synthetic tetrapeptide DEVD-pNa (Asp–Glu–Val–Asp–nitroanilide) as a specific substrate for this enzyme [21]. Samples or blank were placed in a 96-well plate in duplicate. The substrate was added to each well and the plate was incubated at 37°C for 1 h. DEVD-dependent protease activity was assessed by detection of the free *p*-nitroanilide cleaved from the substrates by determining the absorbance at 405 nm.

mRNA gene expression

HO-1 mRNA expression was determined by real time RT-PCR with 18S ribosomal as reference gene. For this purpose, mRNA was isolated from lymphocytes by phenol-chloroform extraction. cDNA was synthesized from 1 μg total RNA using reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. The primers used were: HO-1, forward: 5′-CCA GCG GGC CAG CAA CAA AGT GC-3′ and reverse: 5′-AAG CCT TCA GTG CCC ACG GTA AGG-3′; 18S, forward: 5′-ATG TGA AGT CAC TGT GCC AG-3′ and reverse: 5′-GTG TAA TCC GTC TCC ACA GA-3′; GPx, forward: 5′-TTC CCG TGC AAC CAG TTT G-3′ and reverse: 5′-TTC ACC TCG CAC TTC TCG AA-3′. The PCR conditions were as follows: HO-1, 95°C for 10 min, followed by 40 amplification cycles at 95°C for 0 s, 60°C for 5 s and 72°C for 10 s; ribosomal 18S, 95°C for 10 min, followed by 40 cycles at 95 °C for 10 s, 60°C for 7 s and 72°C for 12 s; GPx, 95°C for 10 min, followed by 40 cycles at 94°C for 1 s, 60°C for 7 s and 72°C for 5 s. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Basal mRNA levels at the beginning of the stage were arbitrarily referred to as 1. The expression of the target gene was normalized with respect to ribosomal 18S.

Nitrite determination

Nitrite levels were determined in lymphocytes by the acidic Griess reaction using a spectrophotometric method. Lysed cells were deproteinized with acetone and kept overnight at −20°C. Samples were centrifuged for 10 min at 15,000*g* at 4°C, and supernatants were recovered. A 96-well plate was loaded with the samples or standard nitrite solutions (100 μl) in duplicate. About 50 μl sulfanilamide (2% w/v) in 5% HCl was added to each well, and 50 μl *N*-(1-napthyl)-ethylenediamine (0.1% w/v) in water was then added. The absorbance was measured at 540 nm following an incubation of 30 min.

iNOS protein levels

iNOS protein levels were determined in neutrophils and lymphocytes by ELISA using polyclonal antibody Anti human iNOS (Stressgen). We followed an adaptation of the previously described method [22].

Suitable dilutions of the neutrophil or lymphocyte suspensions and of the iNOS standard were placed in each well of the plate per duplicate (Polystyrene Assay Plate, Costar). The plate was then incubated at 37°C for 3 h. A solution of 1% bovine albumin was added into each well and the plate was incubated (37°C for 3 h) in order to saturate all binding protein sites of the plate. The plate was then washed four times with NaCl 0.9%-Tween 20. The commercial antibody (diluted 1000 fold) was placed into each well and the plate was newly incubated for 3 h at 37°C. The plate was then washed as above. The secondary antibody against the IgG chain, conjugated to alkaline phosphatase (diluted 500 fold), was placed into each well and the plate was incubated in the same conditions as above. The wells were newly washed and the phosphatase substrate solution was added. Finally, absorbance was measured at 405 nm.

Hydrogen peroxide production

H_2O_2 production in lymphocytes was measured before and after stimulation with phorbol myristate acetate (PMA) using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as indicator.

A stock solution of DCFH-DA (1 mg/ml) in ethanol and PMA (1 mg/ml) in DMSO were prepared, and stored at −20°C until analysis. DCFH-DA (30 μg/ml) in PBS was added to a 96-well microplate containing 50 μl lymphocyte suspension. PMA (3 μM) prepared in HBSS or HBSS alone was added to the wells and the fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37°C for 1 h in FLx800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc.).

MDA determination

MDA as a marker of lipid peroxidation was analyzed in lymphocytes by a colorimetric assay kit (Calbiochem, San Diego, CA, USA). The method used is specific for MDA determination.

Chemiluminescence assay

Opsonized zymosan (OZ) was used as neutrophil stimulant. Zymosan A (Sigma) was suspended in HBSS at a concentration of 1 mg/ml and incubated with 10% human serum at 37°C for 30 min to opsonize the zymosan, followed by centrifugation at 750*g* for 10 min at 4°C. The precipitate was washed twice in HBSS and finally resuspended in HBSS at 1 mg/ml.

OZ suspension (100 μl) was added to a 96-well microplate containing 50 μl neutrophil suspension and 50 μl luminol solution (2 mM in PBS, pH 7.4). Chemiluminescence was measured at 37°C for 90 min in FLx800 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc.). Each sample was determined in duplicate.

Statistical analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 11.0 for Windows). Results are expressed as mean ± SEM. and $P < 0.05$ was considered statistically significant. The statistical significance of the data was assessed by a one-way ANOVA.

Results

In the scuba diving immersion the divers presented a mean 85.5 pulses/min, with a maximal heart rate of 124 (data not shown). In order to evidence the presence of cellular damage induced by the scuba diving session we measured tissue damage markers (Table I). Serum CPK activity–a marker for muscle protein release–and LDH activity–a hemolysis marker–significantly rose after diving and remained high during recovery. Scuba diving induced cellular damage, probably in muscle and in erythrocytes, during immersion. We studied whether scuba diving also affected lymphocyte viability. For this purpose caspase-3 activity, as a marker of apoptosis, and lymphocyte counts were measured (Table II). Both parameters maintained basal values after diving and recovery, suggesting lymphocyte viability was not affected by the scuba session. The increased oxidative stress could induce the activation of antioxidant defenses in lymphocytes to protect themselves against oxidative damage after the diving session. Therefore, we measured the activities of antioxidant enzymes, such as catalase and GPx, involved in free radical scavenging (Table II) and the expression of HO-1 (Figure 2). CAT activity significantly increased about 30% at recovery. Glutathione peroxidase rose significantly after diving, about 21%, and maintained this increased activity during recovery. The levels of HO-1 mRNA rose as a result of the scuba diving session (Figure 2). The HO-1 mRNA increase was only statistically significant after 3 h of recovery.

The antioxidant response of lymphocyte could be related to an increased production of ROS. We therefore investigated lymphocyte capability to produce hydrogen peroxide (Figure 3). Lymphocytes produced H_2O_2 when incubated in the presence of nutrients such as glucose. The scuba diving session induced changes in the lymphocyte capability to produce H_2O_2. This H_2O_2 production rose about 1.2-fold during recovery, both with and without PMA activation. The high H_2O_2 production was observed after diving, when divers were in the open air under normobaric conditions. When the lymphocytes were activated with PMA the H_2O_2 production increased about 6-fold.

Table I. Serum markers of cellular damage in response to a scuba diving session.

	Before	After	Recovery
LDH			
U/l	241 ± 8	274 ± 7*	279 ± 8*
CPK			
U/l	173 ± 20	291 ± 34*	319 ± 33*

One-way ANOVA (*) Significant differences with respect to the before values. $p < 0.05$. Mean ± SEM.

Table II. Effects of a scuba diving session on the lymphocyte number and enzyme activities.

	Before	After	Recovery
Lymphocytes			
10^3 cells/μl	2.50 ± 0.15	2.23 ± 0.08	2.24 ± 0.14
Catalase			
K/10^9 cells	21.4 ± 1.1	23.1 ± 1.1	27.9 ± 2.2*
GPx			
nKat/10^9 cells	75.5 ± 3.9	91.7 ± 5.7*	89.2 ± 4.2*
Caspase-3			
U/10^6 cells	137 ± 9	155 ± 16	146 ± 16

One-way ANOVA (*) Significant differences with respect to the before values. $p < 0.05$. Mean ± SEM.

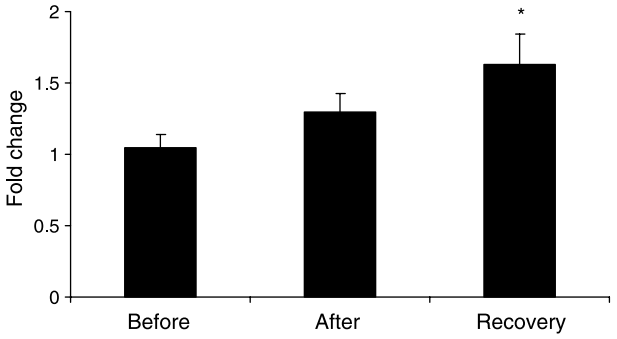

Figure 2. Changes in lymphocyte HO-1 gene expression after a scuba diving session. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. *t*-student for paired data; (*) indicates significant differences between before and after, $p < 0.05$. Mean ± SEM.

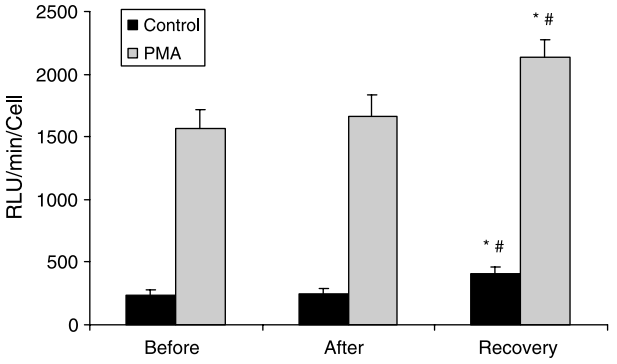

Figure 3. Effects of a scuba diving session on lymphocyte H_2O_2 production. H_2O_2 production in control and in PMA activated lymphocytes, before and after a HBO session. One-way ANOVA (⋆) significant differences with respect to the before values. (#) Significant differences between after and recovery, $p < 0.05$. Mean ± SEM.

We also investigated the neutrophil response to scuba diving, presented in Table III. Neutrophil counts significantly increased during recovery. Neutrophil priming and oxidative activation was determined measuring MPO activity and ROS production, using luminol chemiluminescence. MPO activity significantly decreased about 36% at recovery. HClO production after zymosan activation of neutrophils decreased after diving (29%) and this decrease was greater during recovery (48%).

There is evidence of a relationship between iNOS activity and the expression and activity of some antioxidant enzymes, such as superoxide dismutase, during physical activity. We measured lymphocyte iNOS protein levels and nitrite concentration (Figure 4). Significant increases in lymphocyte iNOS levels (about 38%) and nitrite levels (about 46%) were evidenced after 3 h of recovery compared to basal values, however immediately after diving the levels were similar to the basal value.

In order to identify if HBO alone is enough to induce some of the previously observed changes we measured

Table III. Changes in the neutrophil number and oxidative capability in response to a scuba diving session.

	Before	After	Recovery
Neutrophils			
$10^3/\mu l$	3.24 ± 0.24	3.91 ± 0.33	5.99 ± 0.51*#
MPO			
nKat/10^6 cells	171 ± 12	148 ± 9	109 ± 11*#
Luminol chemiluminescence			
RLU/10^6 cells	904 ± 69	642 ± 60⋆	467 ± 45*#
Temps max (min)	17.2 ± 1.1	17.5 ± 0.9	14.6 ± 1.3

One-way ANOVA. (⋆) Significant differences with respect to the before values. (#) Significant differences between after and recovery, $p < 0.05$. Mean ± SEM.

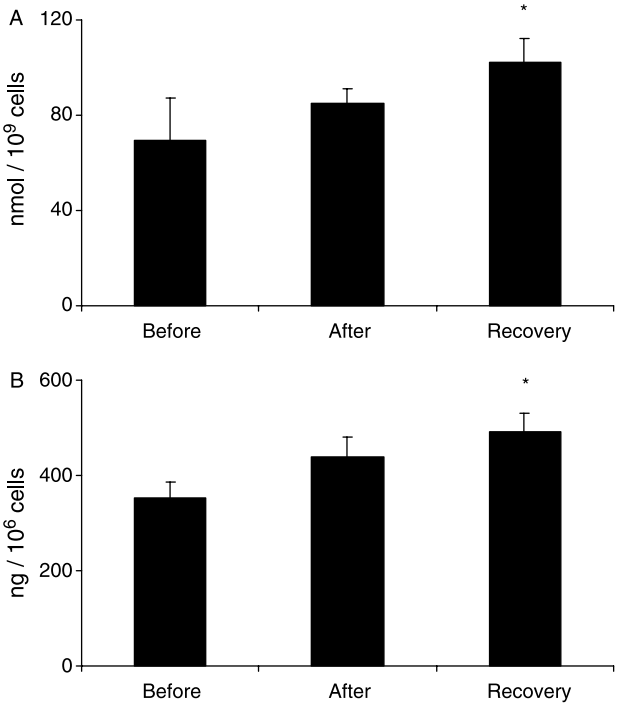

Figure 4. Changes in lymphocyte nitrite (A) and iNOS protein levels (B) in response to a scuba diving session. One-way ANOVA (⋆) significant differences between before and after. (#) Significant differences between before and recovery, $p < 0.05$. Mean ± SEM.

the same parameters after an HBO treatment at resting conditions in another experience. Tissue damage markers CPK and LDH did not increase as a result of the HBO treatment, as seen in Table IV. Lymphocyte count (Table V) was maintained after the treatment, indicating that lymphocyte viability was also unaffected by the hyperbaric exposure. Although no evidence of oxidative stress in the lymphocyte was shown, measured as MDA levels, the antioxidant enzymes responded to HBO exposure. While lymphocyte catalase activity was maintained, glutathione peroxidase increased significantly its activity after the HBO treatment (Table V). The increase in GPx activity did not correspond with a rise in the levels of GPx mRNA, suggesting a post-transcriptional activation (Figure 5(A)). However, HO-1 gene expression was induced by the HBO exposure, rising its mRNA levels about 3-fold after the treatment (Figure 5(B)). As shown with the scuba diving session, the HBO exposure also increased the lymphocyte capability to

Table IV. Serum markers of cellular damage in response to an HBO treatment.

	Before	After
LDH		
U/l	274 ± 7	276 ± 9
CPK		
U/l	126 ± 21	133 ± 24

One-way ANOVA. (⋆) Significant differences with respect to the before values, $p < 0.05$. Mean ± SEM.

Table V. Effects of an HBO treatment on the lymphocyte number, oxidative damage and enzyme activities.

	Before	After
Lymphocytes		
10^3 cells/μl	1.98 ± 0.21	1.68 ± 0.08
Catalase		
K/10^9 cells	22.2 ± 2.4	26.2 ± 2.2
GPx		
nKat/10^9 cells	74.5 ± 5.2	87.3 ± 2.6*
MDA		
μmol/10^9 cells	1.31 ± 0.20	1.54 ± 0.22
iNOS levels		
ng/10^6 cells	247 ± 42	366 ± 47

One-way ANOVA. (*) Significant differences with respect to the before values, $p < 0.05$. Mean ± SEM.

produce ROS. H_2O_2 production increased after the HBO treatment both in basal conditions and when lymphocytes were activated with PMA (Figure 6). iNOS levels tended to be higher after the HBO treatment (Table V), in accordance with the results observed after the scuba diving session, though this increase was not statistically significant.

Neutrophil response to HBO exposure was attenuated when compared to the scuba diving situation. Neutrophil counts were maintained after the treatment; MPO activity and ROS production measured as chemiluminescence production after zymosan activation did not change either (Table VI).

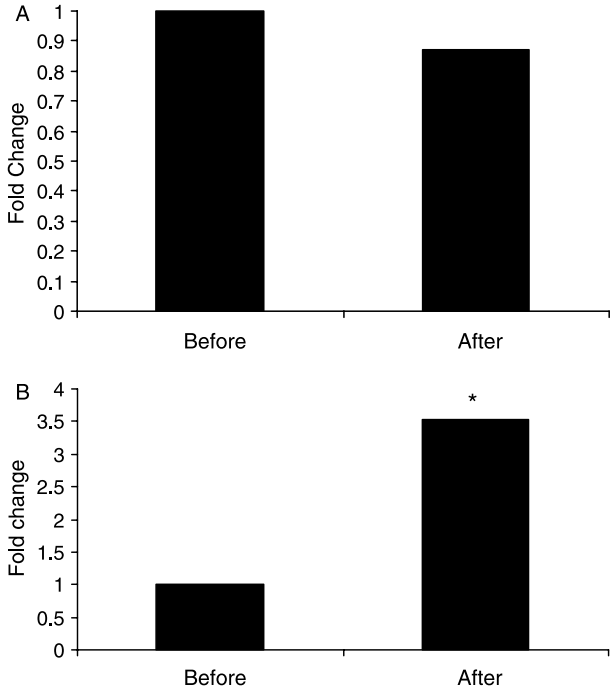

Figure 5. Effects of an HBO exposure on lymphocyte GPx (A) and HO-1 (B) gene expression. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. *t*-student for paired data; (*) indicates significant differences between before and after, $p < 0.05$. Mean ± SEM.

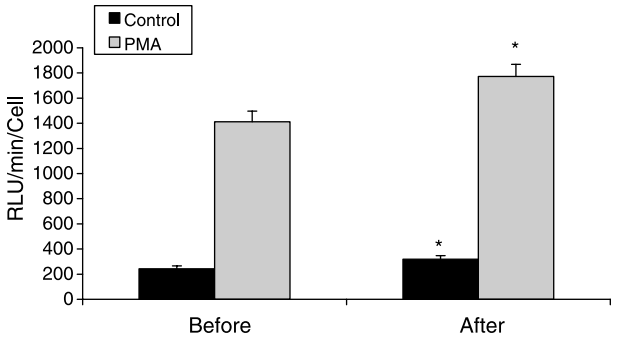

Figure 6. Effects of an HBO treatment on lymphocyte H_2O_2 production. H_2O_2 production in control and in PMA activated lymphocytes, before and after a HBO session. One-way ANOVA (*) significant differences with respect to the before values, $p < 0.05$. Mean ± SEM.

Discussion

Scuba diving combines a situation of physical activity with hyperbaria and high oxygen availability. These situations both lead to an increase in ROS production [23,24] as a result of several processes such as electron leakage from the mitochondrial respiratory chain. Increased oxygen pressure has been successfully used in decompression sickness because it reduces the size of the gas bubbles and increases the amount of oxygen dissolved in plasma and tissues. However this increased oxygen could also facilitate free radical production and tissue damage may occur. Previous studies showed that HBO therapy induces dramatic alterations of PMN functions in normal volunteers, increasing the oxidative burst which could participate in the induction of oxidative damage [25]. In the present study, a short immersion for 10 min at a depth of 40 m is enough to induce changes in lymphocyte and neutrophil pro- and antioxidant status, and to induce a situation of oxidative stress as evidenced with the increase in CPK and LDH serum activities. Increased serum CPK after scuba diving could reflect muscle injury. The low values of the maximal heart rate and the mean heart rate measured in divers during the scuba diving session indicate exercise of moderate intensity. The increase in CPK and LDH serum activities was observed after the scuba diving session, but not after the HBO treatment,

Table VI. Neutrophil number and oxidative capability after an HBO exposure.

	Before	After
Neutrophils		
10^3/μl	3.99 ± 0.49	3.95 ± 0.38
MPO		
nKat/10^6 cells	276 ± 25	255 ± 25
Luminol chemiluminescence		
RLU/10^6 cells	914 ± 82	898 ± 47
Temps max (min)	25.2 ± 0.8	26.3 ± 1.2

One-way ANOVA. (*) Significant differences with respect to the before values, $p < 0.05$. Mean ± SEM.

suggesting that this muscle injury was due to the combination of the moderate physical activity carried out during the immersion and hyperbaria. Injurious and non-injurious exercise produce neutrophil accumulation in muscle after exercise [26]. Invading neutrophils could phagocyte cellular debris and release growth factors that recruit other inflammatory cells such as macrophages which are involved in removing residual cell fragments and in reconstructing muscle fiber [27,28]. In accordance with this data we observed an increase in the circulating neutrophils count only after the scuba diving session, when there was evidence of muscle injury, and not after the HBO exposure. The decrease in the neutrophil oxidative response induced by zymosan, also observed only after the scuba diving session, could be related to a protective mechanism when faced with possible neutrophil recruitment by the damaged tissues. The combination of hyperoxia and physical activity as a consequence of scuba diving could temporally inhibit the oxidative machinery of neutrophils in order to decrease sensitivity against the stimuli, avoiding autoimmune responses and facilitating muscle repair.

Lymphocyte capability to produce H_2O_2 rose after scuba diving and after the HBO treatment and could contribute to the oxidative stress situation. HBO exposure probably resulted in a more pronounced formation of ROS, since the leakage of ROS from mitochondria is believed to increase in direct proportion to the rise in O_2 pressure [29]. Since we measured H_2O_2 production back in normobaric conditions, we suggest HBO exposure could induce some mitochondrial changes that were sustained after returning to normobaric conditions. It has been evidenced that the treatment of HL-60 cells with HBO enhances the intracellular accumulation of H_2O_2 and increases spontaneous and stimulus-induced cell apoptosis in a time-dependent manner [30].

In this study, we found HBO exposure induced the activation of lymphocyte antioxidant defenses in order to protect the cells against the induction of oxidative damage. Several studies have shown that a single HBO exposure induces adaptive protection against further induction of oxidative DNA damage [31,32]. Adaptive protection after HBO seems to be due to enhanced scavenging of ROS distant from nuclear DNA or increased sequestration of transition metals [31]. The oral administration of SOD protects against DNA damage produced after an HBO treatment [33]. CAT activity rose in lymphocytes as a consequence of scuba diving during the recovery period while GPx activity rose both immediately after the scuba diving session and after the HBO exposure at resting conditions. These results suggest that the increased levels of ROS are capable to activate the antioxidant machinery of lymphocytes, and that GPx is one of the firsts antioxidant systems activated to detoxify ROS. This increase may indicate a direct activation or an induction of the expression of these enzymes by ROS or cytokines so as to increase lymphocyte antioxidant defenses [34]. However in this study the hyperbaric condition did not modify the GPx expression, indicating a post-transcriptional regulatory mechanism for this enzyme. Accumulating evidence suggests that HO-1 plays an important role in cellular protection against oxidant-mediated cell injury [35]. Previous studies on HBO indicated clearly increased levels of HO-1 in lymphocytes of volunteers 24 h after HBO treatment (1 h at 1.5 bar) [36]. It seems that the protective effects of HO-1 are related to the formation of the antioxidant molecules bilirubin and iron resulting from heme degradation [5]. Both the scuba diving session at 40 m depth for 10 min and the HBO exposure at 2.2 ATA for 60 min were enough to induce the expression of the HO-1 gene.

Activation of caspase-3 is one biomarker of cell death or apoptosis. Activated caspase-3 drives the apoptotic process by degrading a panel of cellular proteins that are crucial for cell survival. In the present study, caspase-3 activity remained unchanged, which is in accordance with the maintained lymphocyte count, and no evidence of oxidative damage in the lymphocyte was found, indicating that the antioxidant response of lymphocytes is enough to counteract the increased ROS production induced by both the scuba diving session and HBO exposure.

Lymphocytes present the inducible isoform of nitric oxide synthase (iNOS). iNOS present in lymphocytes produces high-levels of sustained NO synthesis when cells are activated. NO is important as a toxic defense molecule against infectious organisms and also regulates the functional activity, growth and death of many immune cells. When NO is generated at high concentrations it is rapidly oxidized to reactive nitrogen oxide species (RNOS) such as peroxinitrite ($ONOO^-$) which mediate most of the immunological effects. $ONOO^-$ production can be avoided by eliminating the superoxide anion, and it has been demonstrated that SOD expression and activity are up-regulated by NO [37] and there is a correlation between lymphocyte iNOS levels and SOD activity [38]. After scuba diving we found a significant increase in iNOS levels and consequently in NO production, as evidenced with the increased nitrite levels. This increase started immediately after the scuba diving session but was statistically significant only after 3 h of recovery. The same pattern was observed after the HBO treatment, suggesting that the high oxygen availability produced by hyperbaria induces iNOS synthesis and consequently NO production, which can lead the antioxidant response in order to minimize oxidative damage. In fact the administration of NO donors has been seen to lead to the activation of HO-1 [16]. These results are in accordance with previous findings of our group and others who described that iNOS protein induction

after an acute bout of exercise is detected only after 3 h and not immediately after [38] though at the transcriptional level an increase in iNOS mRNA has been detected immediately after exercise [39].

It has been evidenced that, despite ROS being toxic molecules, when produced at a low rate they can be considered as signals and stimulators of cellular defenses [40]. In the present paper we demonstrate that a situation of increased O_2 consumption leads to the activation of antioxidant enzymes such as catalase and glutathione peroxidase, thus supporting this protective role for low dosages of ROS.

In conclusion, the HBO exposure in absence of physical activity seems to be enough to increase lymphocyte H_2O_2 production and enhances lymphocyte antioxidant defenses in order to prevent oxidative damage. The combination of hyperbaria and physical activity present in scuba diving leads to a condition of oxidative stress with muscle injury, increased lymphocyte H_2O_2 production, and an acute phase immune response.

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Manuscript II

Scuba diving increases erythrocyte and plasma antioxidant defenses and spares NO without oxidative damage.

Sureda A, Ferrer MD, Batle JM, Tauler P, Tur JA, Pons A
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Scuba diving increases erythrocyte and plasma antioxidant defenses and spares NO without oxidative damage

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Abstract
Purpose: The aim of the present work was to study the effects of a single scuba diving immersion to high depth on erythrocyte and plasma antioxidant defenses, on erythrocyte cellular damage and on nitric oxide (NO) production. **Methods**: Seven male pre-professional divers performed an immersion at a depth of 40 meters for a total time of 25 minutes. Blood samples were obtained before the diving session after overnight fasting, immediately after diving, and 3 h after the diving session finished. Erythrocytes and plasma fractions were purified. **Results**: No significant differences were found in circulating erythrocytes, bilirubin and in haemoglobin concentration attributed to diving. Haematocrit levels were reduced after diving due to the reduction of erythrocyte size that was maintained after three hours of recovery at the surface. Leukocyte counts significantly increased at recovery (38±4%). In erythrocytes, glutathione peroxidase activity significantly increased (18±4%) at recovery. A rise in plasma catalase activity (38±6%) occurred immediately after diving, returning to basal values after recovery. Plasma superoxide dismutase activity significantly increased (58±7%) during recovery. Markers of oxidative damage such as malondialdehyde and protein carbonyl derivates remained unchanged after diving in both erythrocytes and plasma. Nitrite levels significantly rose in plasma and erythrocytes (85±8% and 52±6% respectively) at recovery. **Conclusion**: Scuba diving session induced an antioxidant response to avoid the appearance of cellular damage and to spare NO in plasma and erythrocytes in order to ameliorate the vasodilator role of NO.

Keywords: Oxidative stress, antioxidants, hyperbaric oxygen, nitric oxide, erythrocytes

Introduction

Exposure to hyperbaric oxygen (HBO) favorably leads to an increase of dissolved oxygen in the blood, and it has been successfully used as an adjunctive therapy for several ischemia/reperfusion injuries, including decompression sickness, acute carbon monoxide intoxication or impaired wound healing (14, 42). HBO can relieve tissue hypoxia, restore oxygen necessary for normal oxidative metabolism and stimulate repair and angiogenesis (36, 49). However, it has been demonstrated that HBO also leads to increased reactive oxygen species (ROS) formation that can cause cellular damage (19, 27). The cellular response to HBO-induced oxidative stress has been mainly investigated in animal models (13, 20) and there are few studies performed in humans (5, 12).

Together, erythrocytes and plasma are the most susceptible blood fractions to suffer from lipid oxidative damage induced by ROS (33). Erythrocytes are susceptible to oxidative damage as a result of the high polyunsaturated free fatty acid content of their membrane and the high cellular concentrations of oxygen and haemoglobin, a potentially powerful promoter of oxidative processes, and because they are unable to repair damaged components by re-synthesis (9). However, erythrocytes contain an elaborate antioxidant defence system that involves antioxidant enzymes that eliminate ROS (33). Antioxidants located in both hydrophilic and lipophilic compartments of plasma as a whole with the antioxidant enzymes are actively involved as a defence system against ROS, which are continuously generated (48).

Scuba diving is characterized by the hyperoxia resulting from hyperbaric exposure during diving and the oxygen availability at high pressure, which both could induce oxidative stress. Diving also involves physical activity which increases the oxygen consumption associated with a rise in the production of reactive oxygen species (ROS). The high production of ROS derived of diving can increase the oxidative stress risk. Scuba diving

requires a decompression prior to return to the surface. Decompression sickness is related to the vasoconstriction together with the generation of nitrogen bubbles. It has been shown that nitric oxide (NO) decreases the amount of vascular bubbles produced by decompression in rats (47), whereas the NO-inhibitor L-NAME increases the amount of circulating bubbles (46). NO could induce the peripherical vasodilatation decreasing the generation of gas bubbles in the main vessels and reducing the pernicious effects of the decompressive sickness (47). NO, mainly synthesized by endothelial cells, diffuses to the lumen where it is either oxidized by oxygen, resulting in the formation of nitrite, or it is taken up by erythrocytes (11). Nitrite is the main end-product of NO metabolism and it is relatively stable molecule under intracellular reducing conditions, and has recently been pointed out as a storage pool for NO synthesis in erythrocytes (17). NO availability is associated to the superoxide anion production because both molecules rapidly reacts to form peroxynitrite, a harmful oxidizing agent (29).

In a previous study we evidenced that the hyperoxia associated to scuba diving leads to a condition of oxidative stress with increased serum markers of cellular damage –creatine kinase (CK) and lactate dehydrogenase (LDH)-, while it did not occur after hyperbaric oxygen therapy (15). We hypothesize that scuba diving (hyperoxia and hyperbaric oxygen) induces responses in the antioxidant defenses in order to maintain the redox balance, preventing oxidative damage in erythrocytes and plasma, and to facilitate NO function. The aim of the present work was to study the effects of a single scuba diving immersion to high depth on the erythrocytes and plasma antioxidant system, on the oxidative damage and on the NO synthesis.

Materials and methods

Subjects and study design

Seven male pre-professional divers (34.5 ± 3.6 years old, body mass index (BMI): 23.1 ± 0.6 Kg/m^2) volunteered to take part in this study. The subjects were non-smoker scuba diving learners and they did not take any antioxidant dietary supplement or any routine medication for one month prior to the study. All participants, at the time of the study, had a valid medical certificate for diving. The protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the CAIB (Palma de Mallorca, Balearic Islands, Spain). Subjects were informed of the purpose and demands of the study before giving their written consent to participate. Previously to the immersion, all sportsmen passed a physical and medical test in the laboratory including an electrocardiographic evaluation, a maximal test using a cycloergometer with heart rate and lactacidemia controls, and a blood test (haematological and serum biochemical parameters) to ensure a good performance to practice this sport. All analyzed subjects passed the medical test. Subjects were instructed to refrain from strenuous exercise, and to ingest their habitual diet without any supplement during the 48 h preceding each laboratory test. Divers performed an immersion at a maximum depth of 40 meters for 25 minutes during which they breathed atmospheric air. The return to the surface was with a decompression of 5 min at a depth of 3 meters.

Experimental procedure

Venous blood samples were obtained from the antecubital vein of divers in suitable vacutainers. Venous blood samples were obtained before the diving session after overnight fasting (basal sample), immediately after diving and 3 h after finishing the diving session. Plasma and erythrocytes were purified. Antioxidant enzyme activities, markers of oxidative damage and nitrite levels were measured in both plasma and erythrocytes. Haematological parameters such as erythrocyte and leukocyte number, and hematocrit and haemoglobin concentration were determined in an automatic flow cytometer analyser Technicon H2 (Bayer) VCS system. Bilirubin concentration was also determined in serum. Haematological parameters and bilirubin concentration were analyzed before the first hour after extraction.

Bilirubin determination

Total bilirubin is defined as the amount of bile pigment in serum or plasma that reacts with diazosulphanilic acid into an acid pH to produce azobilirubin (25). Unconjugated bilirubin is solubilised by an accelerator such as caffeine, organic solvents or surfactants. The endpoint absorbance, measured at 548 nm, is directly proportional to the concentration of total Bilirubin.

Erythrocyte and Plasma Purification

Blood samples were centrifuged at 900xg, 4ºC for 30 min. The plasma was recovered, and the erythrocyte phase at the bottom was washed with PBS and centrifuged as above. Erythrocytes were reconstituted and haemolysed with distilled water in the same volume as plasma. Plasma and erythrocytes were immediately stored at -80ºC until use. All biochemical assays were performed in the following week after samples were obtained and were measured in duplicate.

Antioxidant enzyme activities

Catalase (CAT) activity was measured by the spectrophotometric method of Aebi based on the decomposition of H_2O_2 (1). Glutathione reductase (GR) activity was measured by a modification of the Goldberg and Spooner spectrophotometric method (18). This assay required oxidized glutathione as the substrate. Glutathione peroxidase (GPx) activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler (16). This assay required H_2O_2 and NADPH as substrates and glutathione reductase as enzyme indicator. The capability to detoxify hydroperoxides (GPer) was carried out in the same way as for GPx, but the substrate was cumene hydroperoxide. Superoxide dismutase (SOD) activity was measured by an adaptation of the method of McCord & Fridovich (23). The xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion produced the reduction of cytochrome c, which was monitored at 550nm. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37ºC.

Oxidative damage markers

Malondialdehyde (MDA), as a marker of lipid peroxidation, was analyzed in plasma and erythrocytes by a colorimetric assay kit (Calbiochem®, San Diego, CA, USA) following the manufacturer's instructions.

MDA-protein adducts and protein carbonyls were determined by an enzyme immunoassay (Cell Biolabs, Inc.). Briefly, standards or protein samples (10 μg/mL) were adsorbed onto a 96-well plate and incubated overnight at 4ºC. MDA-protein adducts were probed with an anti-MDA antibody, followed by an HRP conjugated secondary antibody. The protein carbonyls present in the sample or standard were derivatized with dinitrophenylhydrazine (DNPH) to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP conjugated secondary antibody. Quantification was performed by comparing unknown samples with a standard curve of known concentration.

Nitrite determination

Nitrite levels were determined in lymphocytes by the acidic Griess reaction using a spectrophotometric method. Lysed cells were deproteinised with acetone and kept at –20ºC overnight. Samples were centrifuged for 10 min at 15000 g at 4ºC, and supernatants were recovered. A 96-well plate was loaded with the samples or nitrite standard solutions (100 μl) in duplicate. 50 μl sulfanilamide (2% w/v) in 5% HCl was added to each well, and 50 μl *N*-(1-napthyl)-ethylenediamine (0.1% w/v) in water was later added. The absorbance at 540 nm was measured following an incubation of 30 min.

Table 1. Hematological and biochemical parameters

	Before	After	Recovery
Erythrocytes (10^6/μL)	5.02 ± 0.06	4.85 ± 0.08	4.88 ± 0.09
Hemoglobin (gr/dL)	15.1 ± 0.1	14.5 ± 0.2	14.7 ± 0.2
Hematocrit (%)	44.3 ± 0.5	42.0 ± 0.6 *	42.0 ± 0.7 *
VCM (Fl)	88.4 ± 0.7	86.7 ± 0.5 *	86.1 ± 0.6 *
HCM (Pg)	30.2 ± 0.2	30.0 ± 0.3	30.3 ± 0.3
CHCM (g/dl)	34.2 ± 0.2	34.6 ± 0.2	35.1 ± 0.2 *
RDW (%)	13.3 ± 0.1	13.5 ± 0.1	13.5 ± 0.1
Bilirubin (mg/dL)	1.03 ± 0.08	1.02 ± 0.09	1.06 ± 0.08
Leukocytes (10^3/μL)	6.74 ± 0.34	7.08 ± 0.35	9.32 ± 0.6 * #

Hematological and biochemical parameters before and after scuba diving and after three hours of recovery. One-way ANOVA. (*) Significant differences respect to before values, (#) significant differences between after and recovery, $p<0.05$. Values represent mean ± s.e.m.

Statistical analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 14.0 for Windows®). Results are expressed as mean ± S.E.M. and $P<0.05$ was considered statistically significant. The statistical significance of the data was assessed by repeated measures of variance (ANOVA).

Results

Table 1 presents the haematological data obtained in the 7 divers at each experimental time. There were no significant differences in circulating erythrocytes, bilirubin, in blood haemoglobin concentration, in the mean haemoglobin content of erythrocytes (HCM) and in the distribution of erythrocyte sizes (RDW). Haematocrit levels and the mean corpuscular volume of erythrocytes (VCM) were reduced immediately after the dive, and this reduction was maintained after three hours of recovery and the haemoglobin erythrocyte concentration (CHCM) significantly increased during recovery. Leukocyte counts significantly increased at recovery 38±4% ($p<0.05$).

A short dive session of 25 minutes at 40 meters depth was enough to induce erythrocyte and plasma antioxidant adaptations. Erythrocyte and plasma antioxidant enzyme activities are shown in Table 2. Erythrocyte Gper activity significantly increased 18±4% ($p<0.05$) after three hours recovery, whereas the other antioxidant enzyme activities maintained the basal values. There was an increase in plasma CAT activity immediately after diving (38±6 %, $p<0.05$). CAT activity returned to the basal values after recovery. Plasma SOD activity significantly increased during recovery 58±7 % ($p<0.05$).

A short diving session was not enough to induce oxidative damage in erythrocytes and in plasma. All markers – MDA, MDA-protein adducts and protein carbonyl levels - maintained the basal values in all situations (Table 3).

Erythrocyte and plasma nitrite concentration, used as marker of NO production, were presented in Figure 1. Nitrite concentration in both plasma and erythrocyte maintained the basal levels immediately after diving, but significantly increased at recovery (85±8 % and 52±6 % respectively, $p<0.05$).

Discussion

Plasma and erythrocyte antioxidant defenses and nitric oxide production changed as result of a short immersion for 25 minutes at a maximum depth of 40 meters. Scuba diving subjects the organism to hyperbaric hyperoxia which could increase ROS generation and tissue damage may occur. The physiological stress of scuba diving in comparison with dry chambers is greater because of additional factors such us immersion, exercise and cold water. The selected sample and the stressor condition (depth) could limit the generalization of the study. However, an open sea diving at 40 meters requires a very good healthy and physical status. Sedentary or non-trained people are not allowed to perform immersions to high depth. The training status of divers will probably results in a better tolerance to the stress situation, because it has been well established that regular training increases antioxidant basal situation in athletes (2, 3). It was described that diving animals present, in general, higher antioxidant status compared to non-diving mammals (45). This descriptive study has been interpreted as the higher antioxidant status of diving animal in order to prevent tissues exposure to high

Table 2. Erythrocyte and plasma antioxidant enzyme activities

	Before	After	Recovery
Erythrocytes			
CAT (K/10^9 cells)	3.69 ± 0.19	3.25 ± 0.21	3.29 ± 0.25
GPx (nKat/10^9 cells)	11.5 ± 0.5	10.7 ± 0.4	11.1 ± 0.5
GPer (nKat/10^9 cells)	5.68 ± 0.25	6.28 ± 0.26	6.69 ± 0.43 *
GR (nKat/10^9 cells)	4.96 ± 0.30	5.04 ± 0.29	5.18 ± 0.36
SOD (pKat/10^6 cells)	0.87 ± 0.05	0.88 ± 0.04	0.91 ± 0.03
Plasma			
CAT (K/L plasma)	200 ± 18	276 ± 23 *	232 ± 19
GPx (nKat/L plasma)	494 ± 16	541 ± 40	553 ± 45
SOD (pKat/L plasma)	48.9 ± 6.4	72.1 ± 9.5	77.3 ± 9.4 *

Erythrocyte and plasma antioxidant enzymes before and after scuba diving and after three hours of recovery. One-way ANOVA. (*) Significant differences respect to before values, $p<0.05$. Values represent mean ± s.e.m.

Table 3. Oxidative damage markers

	Before	After	Recovery
MDA			
Plasma (μmol/L plasma)	11.8 ± 1.1	10.0 ± 0.9	12.5 ± 0.9
Erythrocytes (nmol/10^9 cells)	13.9 ± 0.7	13.9 ± 0.8	13.2 ± 0.8
Protein carbonyl derivates			
Plasma (nmol/mg prot)	0.66 ± 0.06	0.67 ± 0.05	0.69 ± 0.05
Erythrocytes (nmol/mg prot)	18.7 ± 1.9	18.9 ± 2.0	19.2 ± 1.9
MDA-protein adducts			
Plasma (nmol/mg prot)	0.76 ± 0.06	0.77 ± 0.06	0.73 ± 0.08
Erythrocytes (nmol/mg prot)	2.90 ± 0.21	2.97 ± 0.25	2.99 ± 2.82

MDA measured with a colorimetric assay, and protein carbonyl derivates, and MDA-protein adducts measured with immunological techniques in plasma and erythrocytes before and after scuba diving and after three hours of recovery. No significant differences were evidenced, one-way ANOVA. Values represent mean ± s.e.m.

oxygen levels and to avoid the oxidative stress condition related to increased ROS generation (45). Scuba diving also increases the number of circulating leukocytes, suggesting an inflammation-like response. This rise represents the rapid recruitment of leukocytes from marginated pools, like it occurs after infection or after exercise (7, 35). The increase presented after scuba diving is lower than the rise observed after a mountain cycling stage (34) or after a half-marathon (8), indicating a lower intense acute immune response. However, the increase in leukocyte counts was similar to the described after a cycling stage without significant mountain difficulties (32). An immersion at 40 meters for 25 min was enough to provoke a similar leukocyte mobilization than 4 hours cycling. The scuba diving session significantly reduced the haematocrit value in agreement with previous studies (41). The haematocrit and VCM reduction as a whole with the erythrocytes number maintenance, blood haemoglobin concentration and serum bilirubin levels indicated that erythrocytes changed their size, but without apparent haemolysis. The decrease in erythrocyte volume induced by the high pressure during diving concentrated the haemoglobin into the cells as evidenced by the increased CHCM values.

Erythrocytes are very susceptible to oxidative stress as a result of the high polyunsaturated free fatty acid content of their membrane and the high cellular concentrations of oxygen and haemoglobin (33). The lipid peroxidation process of membranes has been pointed as one of the primary events in oxidative cellular damage and has been shown to be associated with fine structure disturbance and subsequent function loss of biological membranes (4). As mature human erythrocytes do not have nucleus or other organelles, the plasma membrane in these cells is the critical target. Erythrocytes are exposed to ROS that are constantly generated from both internal and external sources even under normal conditions, and they may be targeted for oxidative damage during the hyperbaric hyperoxia induced by scuba diving. Although a direct relation cause-effect has to be demonstrated, the higher Gper activity after diving could be indicative of increased levels of lipid hydroperoxides. We previously evidenced that Gper increased in erythrocytes during the recovery of a cycling stage (37). Erythrocytes cannot synthesize proteins, so that the increase in the maximal activities of the enzymes could be attributed to covalent modifications of proteins or to other protein interactions (37). The increased activity of Gper could be also a result of the activation of the enzyme by ROS or by its own substrate, the lipid hydroperoxides. However, markers of cellular damage were unchanged in erythrocytes indicating that the cellular antioxidant response is enough to avoid the ROS-induced damage. In previous results we evidenced a significant increase in MDA and carbonyl groups, measured with colorimetric assays after a cycling mountain stage (38). To avoid the possibility of a false negative of the colorimetric assays to determine MDA and protein carbonyls we used in the present study more sensitive immunological techniques.

Erythrocytes play a key role in the tissue oxygenation during hypoxia (17). Nitrite is relatively stable end-product of NO metabolism and it has been also pointed out as a storage pool for NO synthesis from erythrocytes (17) that produce vasodilatation into the hypoxic tissues. NO metabolism is influenced by the presence of superoxide anion, which reacts with NO to delivery peroxynitrite. Then, an increase in superoxide anion could induce a decreased availability of NO and could produce vasoconstriction. Previously, we have evidenced that the vitamin C supplementation of the diet increased the erythrocyte NO production induced by hypoxia/reoxygenation (34). Then, the erythrocyte oxidative status could alter the NO

production by the erythrocytes. The physiological exposure to scuba diving will lead to an increase in antioxidant enzyme activities which could result in the preservation of NO function. It was evidenced that increasing the antioxidant power with 4 weeks antioxidant vitamins supplementation attenuates the negative effects of a scuba diving on endothelial function (26). Elevated O_2 tensions above ambient will increase NO production by pulmonary cerebral cortex, endothelial cells and intact lungs (10, 40, 44). Hyperoxia leads to vasoconstriction and this may act as a trigger for an increased NO production. There is an increase in superoxide anion production at the initial stage of hyperbaric oxygenation, resulting in NO inactivation and, thus, vasoconstriction (50). Hyperbaric oxygenation leads to overproduction of nitric oxide, which reverses vasoconstriction (24). We evidenced an increase in NO production 3 hours after scuba diving in both plasma and erythrocytes. The return to normobaria and normoxia needs of vascular vasodilatation, and the NO production is reinforced. Previous studies evidenced an increase in the total antioxidant capacity measured in plasma after breathing oxygen under pressure (22). In our study, CAT and SOD activities significantly increased after scuba diving enhancing the plasma antioxidant status. This rise in antioxidant enzymes could be important in order to avoid oxidative damage induced by ROS overproduccion and to allow the vasodilatory function of NO. It has been showed that one hour after the dive, microbubbles could be detected in the heart chambers of divers (6). The increase in NO during recovery could be important to avoid the potential deleterious effects of air bubbles and to prevent the decompression sickness. In fact, it has been described that hypertensive patients have a reduction of the extracellular SOD activity (51). The increase of plasma SOD could be important in order to avoid the reaction between the superoxide anion and the NO and, consequently, reducing the formation of peroxynitrite. Rousseau et al. (28) evidenced an increase in GP activity and a decrease in glutathione after a submaximal apnea dynamic diving to buffer against the increase of ROS. It was also described an increase in SOD activity in human patients with rheumatoid arthritis under HBO therapy (21). The increased activity of plasma SOD could produce an increased production of hydrogen peroxide in plasma. This point is also counteracted by a similar rise of plasma CAT. The increased activities of these enzymes could be attributed to an increase of their plasma levels in addition to the mechanisms that activate the antioxidant enzymes in erythrocytes. We previously pointed out the possibility to secrete CAT from neutrophils during intense exercise as a part of the acute phase immune response like occurs with myeloperoxidase (31, 32). Then, the increased CAT activity observed in plasma during the recovery of scuba diving could have a neutrophilic origin.

The genotoxic potential of NO is referred to the formation of peroxynitrite from NO and superoxide. It has been evidenced that a single HBO induces oxidative DNA damage (19). The increase of SOD in plasma could be important in order to avoid the reaction between the superoxide anion and the NO and, consequently, reducing the formation of peroxynitrite. The role of extracellular SOD, as protector against oxidative stress, could be related to an attenuation of renal p22 (phox) expression, NADPH oxidase activation and the accompanying renal vasoconstriction and hypertension (43). However, it can also take into account that EC-SOD spares NO and then, the NO function is enhanced. The physiological relevance of elevations in NO concentration due to hyperoxia requires more investigation. These changes may contribute to angiogenesis augmentation and inhibition of neutrophil β_2-integrin function that have been reported with hyperbaric O_2 (30, 39).

Plasma and erythrocytes antioxidant system shows a response to scuba diving session at 40 meters depth increasing the ROS detoxifying enzymes in order to avoid the appearance of oxidative damage. These adaptations also spare NO in order to facilitate its vasodilatory function.

Acknowledgments

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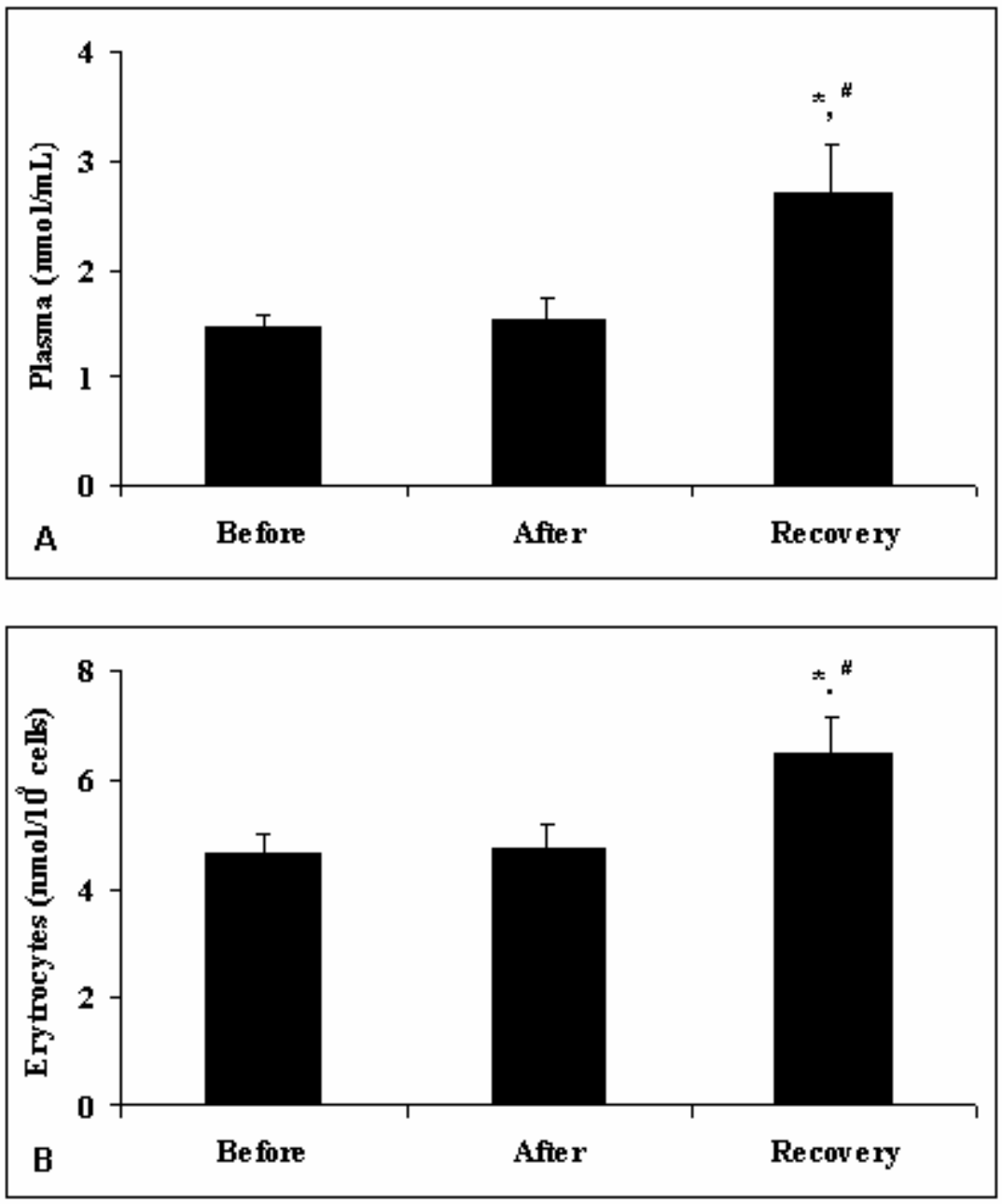
FIGURES

Figure 1. Plasma and erythrocyte nitrite concentration.



Nitrite levels before and after scuba diving and after three hours of recovery. One-way ANOVA. (*) Significant differences respect to before values. (#) significant differences between after and recovery, $p<0.05$. Values represent mean ± s.e.m.

Manuscript III

Intense physical activity enhances neutrophil antioxidant enzyme gene expresión. Immunocytochemistry evidence for catalase secretion.

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Intense physical activity enhances neutrophil antioxidant enzyme gene expression. Immunocytochemistry evidence for catalase secretion

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Abstract

We studied the effects of intense exercise on the neutrophil antioxidant enzyme activities and gene expression. Blood samples were taken from seven cyclists in basal conditions and 3 h after two competition stages of 165 km. Serum creatine kinase (CK) activity, plasma carbonyl derivatives and uric acid levels increased after exercise. The cycling stage induced neutrophilia and increased myeloperoxidase (MPO) activity and reactive oxygen species (ROS) production. Antioxidant enzyme activities (catalase, glutathione peroxidase and superoxide dismutase) decreased after exercise, although gene expression increased. Immunocytochemistry showed catalase (CAT) enzyme equally distributed between the cytoplasm and organelles before exercise, and after exercise the cytoplasmic CAT levels were reduced and were absent in the compartments. After *in vitro* stimulation with opsonized zymosan (OZ) the extracellular CAT levels increased. This suggests a CAT secretion in order to avoid neutrophil-induced oxidative damage at a local level or to regulate the function of ROS as extracellular signalling molecules.

Keywords: *Oxidative stress, antioxidants, exercise, catalase, gene expression*

Abbreviations: *BSA, Bovine serum albumin; CAT, catalase; CK, creatine kinase; DNPH, 2,4-dinitrophenylhydrazine; GP, glutathione peroxidase; HBSS, Hank's balanced salt solution; MDA, malondialdehyde; MPO, myeloperoxidase; OZ, opsonized zymosan; ROS, reactive oxygen species; SOD, superoxide dismutase*

Introduction

Moderate exercise and regular training enhance the immune function. However intense exercise affects the immune system, inducing oxidative stress and increasing the risk for upper respiratory tract infections [1]. During exercise, oxygen consumption is increased and the reactive oxygen species (ROS) production is augmented due to the mitochondrial electron transport flux [2]. Although the cellular antioxidant defence presents a great adaptation to acute and chronic exercise, intense exercise is associated with increases in lipid peroxidation and protein oxidation as well as the release of muscle enzymes [3–5].

Exercise has been shown to induce inflammatory-like changes in the immune cell that resemble the acute immune phase response to infection. Physical activity induces neutrophil priming for oxidative activity and activates acute phase protein release [6]. (Luminol)-dependent chemiluminescence response of

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the neutrophils, which indicates myeloperoxidase (MPO)-mediated formation of highly reactive oxidants, is enhanced after exercise [7]. However, strenuous exercise can inhibit the neutrophil capability to generate oxygen radicals [8]. In a previous work, we showed a delay in neutrophils chemiluminescence after repetitive diving apnea sessions [9]. This neutrophil priming is closely associated with the exercise-induced mobilization of neutrophils from the marginated pool into blood circulation, which is mediated by the overshooting of catecholamines during exercise, and can be regulated by signalling factors secreted by endothelial cells [10]. The neutrophil proteins that appear in blood during degranulation can be involved in enhancing the bactericidal potency of blood, neutrophil efflux from bone marrow, and the conditioning of blood endothelium for leukocyte extravasation [11].

The toxicity of ROS produced by neutrophils could damage the neutrophil itself and adjacent tissues contributing to the oxidative stress situation [12,13]. The antioxidant system involves enzymes such as superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GP) that act by detoxifying the ROS generated. SOD is important for the protection of activated neutrophils from superoxide generated by NADPH oxidase on the cell membrane. Neutrophils contain the highest CAT activity of all phagocytes [14]. This high activity may have a profound role during the respiratory burst and oxidative stress of activated neutrophils, providing high resistance to exogenous H_2O_2 [14]. Glutathione metabolism may also play an important role in the protection against exogenous oxidants [15]. Antioxidant enzymes are known to be regulated by inflammatory cytokines, oxygen tension and ROS in human neutrophils [14]. ROS, when generated at sub-toxic levels, can mediate or enhance diverse intracellular signal transduction pathways [16,17]. The general response after exercise includes an increase of some cytokines and several hormones as well as ROS production that could induce the antioxidant enzyme synthesis [18]. However, we have evidenced a decrease in neutrophil antioxidant enzyme activities as well as protein levels after exhaustive exercise [19–21] and after diving apnea sessions [22]. This decrease could be explained by different mechanisms such as accelerated enzyme turnover, enzyme inactivation by the ROS themselves and antioxidant enzyme release from neutrophil to enhance plasma antioxidant defences.

In this work, we studied the neutrophil oxidative capability and the existence of cellular oxidative damage on proteins and lipids after an intense exercise. We observed a decrease in antioxidant enzyme activities despite an increase in the corresponding mRNA content. We support the hypothesis that relocalization to the extracellular space could contribute to the reported changes, at least in the case of CAT.

Materials and methods

Subjects and study design

Seven voluntary male subjects participated in this study. They were all professional cyclists. The study took place in the Mallorca Challenge 2003 (Balearic Islands), a five-day competition for professional cyclists. The sportsmen's mean ($\pm$ SEM) age was 23.8 ± 0.9 years, and weight 70.0 ± 1.5 kg. We studied the second and fourth stages. Both stages were similar with 164.5 and 166.3 km, respectively, and without significant mountain difficulties. The cyclists completed the stages in 244 ± 11 min.

For the *in vitro* experiment (see below) the volunteer subject was a sportsman of 23 years and 68 kg of weight. All subjects were informed of the purpose of this study and the possible risks involved before giving their written consent to participate.

Experimental procedure

Venous blood samples were taken from the antecubital vein with suitable vacutainers with EDTA as anticoagulant. Samples were taken to all subjects in the morning previous to the both cycling stages after overnight fasting and 3 h after the end of each stage. Samples were obtained 3 h after exercise when the oxidative damage in blood cells is evident [23]. Neutrophils and plasma were obtained from blood samples. The enzyme activities and gene expression of CAT, SOD, GP and MPO were determined in neutrophils. Protein carbonyl derivatives and malondialdehyde (MDA) were measured as well in neutrophils. Creatine kinase (CK) and uric acid were measured in serum. The oxidative capacity of neutrophils was determined by luminol chemiluminescence.

Neutrophil purification

The neutrophil fraction was purified following a modification of the method described by Boyum [24]. Blood was introduced carefully on Ficoll in a proportion of 1.5:1 and then centrifuged at 900*g*, at 4°C for 30 min. The precipitate containing erythrocytes and neutrophils was incubated at 4°C with ammonium chloride 0.15 M to hemolyze the erythrocytes. The suspension was centrifuged at 750*g*, at 4°C for 15 min and the supernatant was then discarded. The neutrophil phase at the bottom was washed first with ammonium chloride and then with phosphate buffer saline (PBS), pH 7.4. Finally, neutrophils were lysed with distilled water for enzymatic and carbonyl analysis or resuspended in Hank's balanced salt solution (HBSS) for chemiluminescence assays. The water-lysed neutrophils were concentrated three times with respect to blood, while neutrophils for chemiluminescence assay were used in the same concentration as blood. Neutrophils were

quantified in fresh blood using an autoanalyzer system (Technicon DAX System).

Enzymatic determinations

We determined the activities of CAT, SOD, GP and MPO in neutrophils. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37°C.

CAT activity was measured by the spectrophotometric method of Aebi [25] based on the decomposition of H_2O_2. GP activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler [26]. This assay required H_2O_2 as the substrate and NADPH and glutathione reductase as enzyme indicator. Total SOD activity was measured by an adaptation of the method of McCord and Fridovich [27]. The xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion produced the reduction of cytochrome C, which was monitored at 550 nm. The SOD of the sample removed the superoxide anion and inhibited cytochrome C reduction. MPO activity of neutrophils was measured by guaiacol oxidation [28]. The reaction mixture contained sodium phosphate buffer pH 7 and 13.5 mM guaiacol. The reaction was initiated by adding 300 μM H_2O_2, and changes at 470 nm were monitored.

Protein carbonyl derivatives determination

Carbonyl derivatives were measured in plasma and neutrophils by an adaptation of the method of Levine [29]. Samples were deproteinised with trichloroacetic acid. Protein precipitates were resuspended with 2, 4-dinitrophenylhydrazine (DNPH) 10 mM, and incubated for 60 min at 37°C. Samples were then precipitated with 20% trichloroacetic acid, and centrifuged for 10 min at 1000*g* at 4°C. The precipitate was washed twice with ethanol–ethyl acetate (1:1; v/v) to remove free DNPH. Guanidine 6 M in phosphate buffer 2 mM, pH 2.3 was added to the precipitate, and samples were incubated for 40 min at 37°C. Finally, samples were centrifuged for 5 min at 3000*g* at 4°C to clarify the supernatant and absorbance was measured at 360 nm. The molar absorption of 22,000 M^{-1} cm^{-1} was used to quantify the levels of protein carbonyls. Samples were analysed against a blank of guanidine solution.

MDA determination

Plasma and neutrophil MDA levels were determined as a marker of lipid peroxidation which was analyzed by a colorimetric assay kit (Calbiochem, San Diego, CA, USA).

Chemiluminescence assay

Opsonized zymosan (OZ) was used as neutrophil stimulant. Zymosan A (Sigma) was suspended in HBSS at a concentration of 1 mg/ml and incubated with 10% human serum at 37°C for 30 min to opsonize the zymosan, followed by centrifugation at 750*g* for 10 min at 4°C. The precipitate was washed twice in HBSS and finally resuspended in HBSS at 1 mg/ml.

Chemiluminescence assay was performed by an adaptation of the method by Edwards [30]. Luminol is a lumigenic probe which can be oxidized by H_2O_2 and HOCl. Thus, in activated neutrophils, luminol chemiluminescence measures the combined activities of the NADPH oxidase plus MPO. OZ suspension (100 μl) was added to a 96-well microplate containing 50 μl neutrophil suspension and 50 μl luminol solution (2 mM in PBS, pH 7.4). Chemiluminescence was measured at 37°C for 90 min in FLx800 Microplate Fluorescence Reader (Bio-tek Instruments Inc., USA). Each sample was determined in duplicate.

CK and uric acid determinations

These determinations were made using commercial clinical kits in an autoanalyser system (Technicon DAX System).

In the determination of CK activity, the enzyme reacts with creatine phosphate and ADP to form ATP, which is coupled to the hexokinase/GDP reaction generating NADPH [31]. The activity was monitored by measuring the change in absorbance at 340 nm. Uric acid was determined by an enzymatic method based on the specific uricase-catalysed oxidation of uric acid to allantoin and H_2O_2 [32]. The reaction of with 4-aminoantipyrine and 2-hydroxy-3, 5-dichlorobenzene sulphonate catalysed by peroxidase produces a red chromophore, quantified by endpoint at 524 nm.

RNA extraction and relative quantitative RT-PCR assay

Antioxidant enzymes mRNA expression was determined by multiplex real time RT-PCR using human 36B4 rRNA as reference. For this purpose, total RNA was isolated from neutrophils by Tripure extraction (Roche Diagnostics, Germany). RNA (1 μg) from each sample was reverse transcribed using 50 U of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol oligo (dT) for 60 min at 37°C in a 20 μl final volume, according to manufacturer instructions. The resulting cDNA (0.5 μl) was amplified using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics, Germany). Amplification was performed at 55°C melting temperature and 40 cycles (45 for MPO). The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Antioxidant enzyme levels before and after the stage were normalized to the invariant control 36B4 rRNA. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. Primers used are listed in Table I.

Table I. List of gene-specific primers used in PCR.

Genes	Forward primer	Reverse primer	Product size (bp)	GeneBank accession no
hCu/Zn-SOD	AAGGCCGTGTGCGTGCTGAA	CAAGTCTCCAACATGCCTCT	245	AY450286
HMn-SOD	GAGAAGTACCAGGAGGCGTTG	CAAGCCAACCCCAACCTGAGC	252	BC035422.1
HGP	ACATGCCTACAGGTATGCGT	GAGCAGAACAATTGGACCTA	218	NM_002084
HCAT	TTTGGCTACTTTGAGGTCAC	TCCCCATTTGCATTAACCAG	440	NM_001752
HMPO	CCAGGAAGCCCGGAAGAT	CGGAAGGCATTGGTGAAGA	167	NM_000250
h36B4-rib	ATGTGAAGTCACTGTGCCAG	GTGTAATCCGTCTCCACAGA	420	M17885

Immunocytochemistry

CAT localization in neutrophils was determined by immunocytochemical gold-labelling [33]. Cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and washed three times with cacodylate buffer. After that, cells were postfixed with 1% osmium tetroxide and stained with uranyl acetate and lead citrate. Samples were dehydrated and embedded in Epoxy resin. Ultrathin sections were collected on gold grids. Sections were incubated in 10% H_2O_2 to permeabilize the resin. Then, samples were washed five times with distilled water and incubated 30 min with 5 mg/ml bovine serum albumin (BSA) in PBS. Samples were incubated overnight at 4°C with rabbit anti-CAT antibody (40 μg/ml) in 0.05 M Tris buffer, pH 7.2 with 1% BSA. The sections were washed three times with Tris buffer, pH 7.2, containing 0.2% BSA and incubated with the gold-labelled secondary antibody (10-nm gold particles; Sigma) diluted 1:50, 3 h at room temperature. Finally, samples were washed with large volumes of 0.05 M Tris buffer, pH 7.2, containing 0.2% BSA, followed by Tris buffer, pH 7.2, without BSA, and distilled water. Samples were examined using a transmission electron microscope (70 kV).

In vitro *neutrophil activation*

To further study the possible CAT release, neutrophils obtained from one subject in resting conditions were incubated in tetraplicate with OZ.

Zymosan was opsonized by adding 10% plasma to a zymosan dilution 1 mg/ml in PBS and incubating for 30 min at 37°C. OZ was centrifuged at 750*g*, 4°C for 10 min and the precipitated was resuspended in HBSS at 1 mg/ml. Purified neutrophils were resuspended to a final concentration of 10^7 cell/ml in HBSS with 0.2 mg/ml OZ and were incubated at 37°C for 30 min. A negative control without zymosan was also performed in tetraplicate. Aliquots were taken at time 0 and 30 min and centrifuged at 700*g*, 4°C for 10 min. The supernatant was recovered to another vial and the precipitated was resuspended with HBSS in the initial volume and sonicated.

CAT and MPO activities were determined as described above in the supernatant and precipitated fractions.

Statistical analysis

Statistical analyses were carried out using a statistical package for social sciences (SPSS 11.0 for windows). Results were expressed as means ± SEM and $P < 0.05$ was considered statistically significant. *t*-Student for paired data was used to determine the significance of changes, in all parameters measured, induced by the cycling stage.

For the *in vitro* neutrophil activation experiment the statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analyzed were time (*T*), and zymosan activation (*Z*). When significant effects were found, a one-way ANOVA was used to determine the differences between pre and post-exercise.

Results

A cycling stage induces cellular and plasma molecular damage

A group of seven professional cyclists was monitored as described in Material and Methods. In order to

Table II. Oxidative stress markers and uric acid levels in plasma.

	Pre-exercise	Post-exercise	*t*-Student (*p*)
Plasma MDA (μmol/l)	4.62 ± 0.20	5.03 ± 0.28	0.484
Plasma carbonyl derivatives (μmol/l)	345 ± 9	390 ± 13⋆	0.010
CK (U/l)	272 ± 30	343 ± 36⋆	0.046
Uric acid (mg/dl)	3.75 ± 0.21	4.31 ± 0.20⋆	0.043

Effects of the cycling stage on plasma MDA, protein carbonyl derivatives and uric acid levels, and CK activity. *t*-Student for paired data. (⋆) Indicates significant differences between pre and post-exercise ($p < 0.05$). Results are the mean ± SEM, $n = 7$.

Table III. Leukocyte and neutrophil counts and markers of oxidative damage in neutrophils.

	Pre-exercise	Post-exercise	*t*-Student
Leukocytes (10^3/μl blood)	6.84 ± 0.48	9.83 ± 0.45⋆	$p < 0.001$
Neutrophils (10^3/μl blood)	3.44 ± 0.32	6.66 ± 0.41⋆	$p < 0.001$
Neutrophil MDA (μmol/10^9 cells)	0.17 ± 0.02	0.18 ± 0.02	$p = 0.876$
Neutrophil protein carbonyl derivatives (μmol /10^9 cells)	0.67 ± 0.12	0.65 ± 0.08	$p = 0.948$

Effects of the cycling stage on leukocyte and neutrophil number (10^3/μl blood) and markers of oxidative damage in neutrophils expressed per cell number. *t*-Student for paired data. (⋆) Indicates significant differences between pre and post-exercise ($p < 0.05$). Results are the mean ± SEM, $n = 7$.

verify the presence of oxidative unbalance induced by the cycling stage, we measured tissue damage and oxidative stress markers (Table II). Serum CK activity, a marker for muscle protein release, significantly increased after exercise (Table II). The high circulating CK activity could serve as an indicator for the severity of exercise in muscle tissue. On the other hand, no significant changes were reported in plasma MDA at the end of the study, although a slight tendency to increase was observed (Table II). Plasma protein carbonyl levels significantly increased after exercise. Plasma uric acid levels increased as a result of the physical activity (Table II). Uric acid is the end-product of purine nucleotide catabolism during exercise and at the same time this metabolite serves as a free radical scavenger in plasma [34,35].

The increased oxidative burst in neutrophil after a cycling stage is not accompanied with oxidative stress

The cycling stage induced an increase in the number of total circulating leukocytes and in the circulating neutrophils (Table III). As an additional step, we wanted to verify if this increase in the number of circulating neutrophils was accompanied by an oxidative burst. To this purpose, we measured the MPO activity, marker of oxidative capability, in neutrophils before and after cycling stage. As shown in Table IV, MPO activity, expressed as per cell number, significantly increased (20%) after exercise. In addition, neutrophils responded to the cycling stage with a high increase of ROS production (69%) measured by luminol chemiluminescence assay, and reducing the time at which the maximal chemiluminescence level was attained (Figure 1). Neutrophils after exercise significantly advanced the maximum chemiluminescence response to OZ in 462 s vs. pre-exercise neutrophils. Altogether, these data indicate neutrophils are primed to oxidative burst after stimulation induced by the intensity of the exercise.

In a next step, we wanted to measure how the oxidative stress, generated as a consequence of neutrophil function, could affect the oxidant status of these cells. To this end, we determined MDA and protein carbonyl derivatives, markers for oxidative damage, in neutrophils. As shown in Table III, these parameters were unchanged in neutrophils after the cycling stage.

Table IV. Effects of the cycling stage on neutrophil antioxidant enzyme and MPO activities.

	Pre-exercise	Post-exercise	*t*-Student
Catalase (K/10^9 cells)	15.6 ± 2.0	10.3 ± 0.6⋆	$p = 0.022$
GP (nKat/10^9 cells)	49.6 ± 4.9	36.0 ± 2.0⋆	$p = 0.018$
SOD (pKat/10^9 cells)	12.3 ± 1.1	6.47 ± 0.38⋆	$p < 0.001$
MPO (nKat/10^6 cells)	137 ± 9	166 ± 9⋆	$p = 0.045$

Effects of the cycling stage on neutrophils antioxidants enzyme activities and MPO activity expressed per cell number. *t*-Student for paired data. (⋆) Indicates significant differences between pre and post-exercise ($p < 0.05$). Results are the mean ± SEM, $n = 7$.

Antioxidant enzymes decrease their activities in neutrophil after a cycling stage though their gene expressions increase. Catalase mobilizes from cytoplasm

The absence of oxidative damage could be related to the activation of antioxidant defences in neutrophils to protect themselves against oxidative damage induced after physical exercise. Therefore, we measured the activities of antioxidant enzymes involved in free radical scavenging. Table IV shows that all three antioxidant enzymes displayed the same response pattern, decreasing their activities after exercise when expressed per cell number: CAT (51%), GP (37%) and SOD (47%).

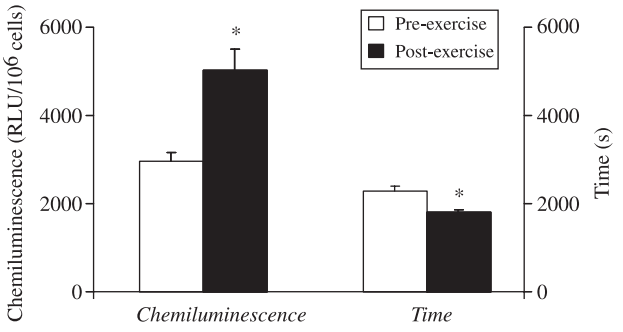

Figure 1. Effects of the cycling stage on neutrophil luminol chemiluminescence. Luminol chemiluminescence (RLU/10^6 cells) and the time of the maximum RLU (s) before and after the cycling stage. *t*-Student for paired data. (⋆) Indicates significant differences between pre and post-exercise, $n = 7$.

Table V. mRNA antioxidant enzyme levels.

	Fold induction	*t*-Student
Catalase	3.60 ± 0.78⋆	0.046
GP	1.73 ± 0.42⋆	0.044
Cu-Zn-SOD	1.48 ± 0.29⋆	0.043
Mn-SOD	3.21 ± 1.08	0.404
MPO	+	

Effects of the cycling stage on antioxidant enzyme and MPO gene expression. *t*-Student for paired data. (⋆) Indicates significant differences between pre and post-exercise. See Materials and Methods for calculations. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. The (+) in the MPO mRNA indicates induction but no quantification due to no detection of the transcript at the beginning of the stage. Only two cyclists displayed MPO expression before the stage with a 3-fold increase, $n = 7$.

This lower activity could be explained by a decrease in the level of gene expression for these enzymes. However, as shown in Table V, the level of the corresponding mRNAs was higher at the end of the stage for all tested genes, indicating that the decrease in enzyme activity could not be related to changes at the level of gene expression. In order to find alternative explanations, we proceeded to study the intracellular location for the case of CAT. Figure 2 shows an electron microscopy image of immunogold labelled CAT. The enzyme was located in the cytoplasm as well as in vesicle-like compartments before the cycling stage. However, after exercise the enzyme levels were reduced in cytoplasm and no marked vesicles were evidenced. Altogether, these results are in agreement with either a possible release of the enzyme from neutrophils to the plasma or alternatively an accelerated rate of degradation in specific cell compartments (i.e. fagosomes, lysosomes).

In vitro *neutrophil activation with opsonized zymosan induces catalase release*

To discriminate between CAT release or degradation, we studied the *in vitro* neutrophil response to activation with zymosan. In basal conditions approximately the 15% of total CAT determined was found in the supernatant (Figure 3A). After the 30 min incubation with zymosan extracellular CAT increased about 7.5%, being significantly higher in the activated group than in the non-activated group. In order to discard that the high basal levels of extracellular CAT were due to lysis we also determined the percentage of extracellular MPO activity, shown in Figure 3B. Only about 1% of total MPO activity was found in supernatant in basal conditions before the incubation, so the high levels of CAT cannot be ascribed to neutrophil lysis. An effect of both the time and activation factors was found in the percentage of extracellular MPO activity before and after the incubation with zymosan. The percentage of MPO activity in the supernatant after 30 min of incubation with OZ was significantly increased vs. the basal condition prior to incubation (1.3%) and vs. the non-activated group after the 30 min (0.9%).

Discussion

Exercise leads to an increase in metabolic oxidative processes, accompanied by ROS generation, and tissue damage, evidenced by intracellular enzyme leakage. CK is found almost exclusively in the muscle tissue, and is used as the most common marker of muscle damage. The cycling stage increases CK activity in serum, indicating specifically muscle damage. However, the magnitude of CK levels in serum is lower than other reports in which muscle damage produce CK values as high as 80,000 U/L [36]. The muscle damage would be higher than expressed by the CK values at 3 h after exercise because CK takes many hours to reach the circulation through the lymphatic system.

The increase in protein carbonyl levels after the cycling stage indicates that the blood antioxidant system is not sufficient to avoid the plasma protein

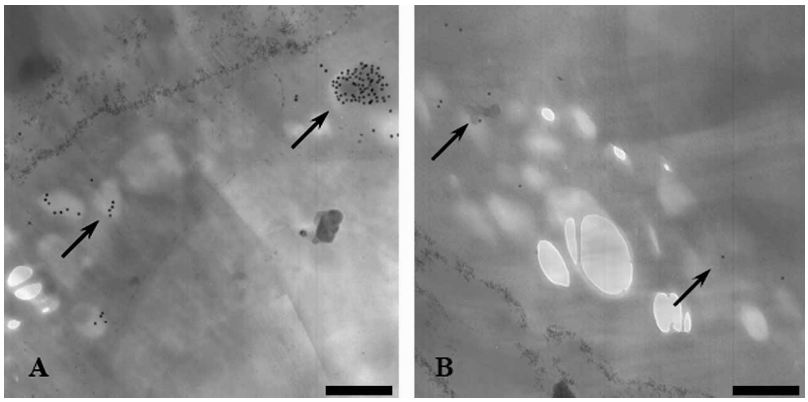

Figure 2. Effects of the cycling stage on neutrophil CAT localization. Immunogold localization of CAT (arrows). Representative electron micrograph of a region of neutrophil cytoplasm before (A) and after (B) exercise. Cells were incubated with cationized ferritin as a marker of plasma membrane. (Original magnification × 50,000; bar = 0.2 μm.)

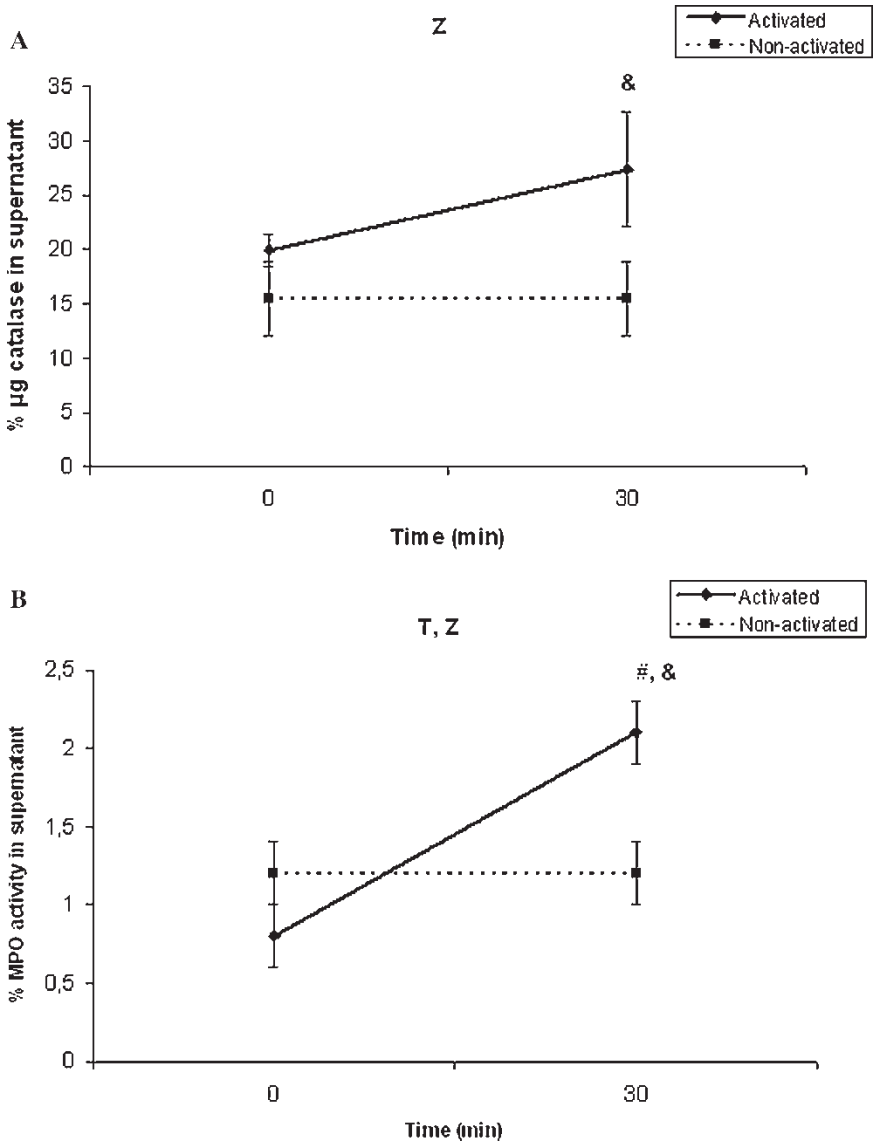

Figure 3. Percentage of CAT (A) and MPO activity (B) found in supernatant before and after a 30 min incubation of neutrophils with OZ. Statistical analysis: Two-way ANOVA. (*Z*) indicates significant effects of Zymosan activation. (*T*) indicates significant effects of time. ($T \times Z$) indicates significant interaction between the two factors. No significant interactions were evidenced. (&) indicates significant differences between activated and non-activated groups. (#) indicates significant differences between pre-incubation and post-incubation ($p < 0.05$), $n = 4$.

damage induced by the cycling stage. The uric acid levels increase in plasma after exercise as a result of increased degradation of adenine nucleotides and the transformation of xanthine dehydrogenase into xanthine oxidase [37]. Uric acid released to the blood has antioxidant properties and could contribute to the blood antioxidant defences. Our findings are in accordance with previous studies reporting increased antioxidant enzymes [38], and antioxidant nutrients [39,40] in plasma in response to extreme exercise.

The cycling stage induces an increase in the number of circulating neutrophils as has been evidenced in other physical activity sessions [20–22]. The increase in the number of neutrophils is related to the intensity of the physical activity [41]. We evidenced that an exhaustive exercise such as a duathlon competition or a cycling mountain stage increases about 4-fold the neutrophil counts [19,20], while in our study, a flat cycling stage neutrophils number only increases 2-fold. Circulating neutrophils after the cycling stage are primed for oxidative burst as is evidenced by the heightened MPO activity, the rapid response to zymosan stimulation and the increase in the maximum luminol chemiluminescence. The typical features of this immune response are the release of neutrophil granule constituents, such as MPO, lactoferrin or elastase [11,42], the decrease of antioxidant enzyme activities [19,21,43] and stimulation of phagocytosis as well as other innate mechanisms [44]. The mechanisms underlying these exercise-induced

immunological changes are multifactorial and have been attributed to the release of immunomodulating stress hormones (catecholamines and cortisol) or local muscle damage [45].

The mechanism to explain exercise-induced muscle damage and repair is not well defined. Damaged muscle releases several proteins to the blood that activate a wide range of defensive reactions similar to the acute-phase immune response [46,47]. This response is important for its antiviral and antibacterial actions as well as for promoting the clearance of damaged tissue and subsequent repair [48]. Within hours of injury or exercise neutrophils migrate to the site of injury, where they phagocyte tissue debris and release factors such as lysozyme and oxygen radicals that are known to increase protein breakdown. It is reported that neutrophil infiltration is maximal after the time of peak plasma CK and it is likely that in exercised muscle infiltrating neutrophils act to scavenge cellular debris rather than to cause damage to the muscle [36,49]. It is probable that the neutrophil priming for oxidative burst could be related with their role in muscle repair.

Neutrophils produce ROS during phagocytosis or by stimulation with a wide variety of agents through the activation of NADPH oxidase that is assembled at the plasma membrane. The increased ability to produce ROS in activated neutrophils could induce oxidative damage to themselves. However, markers of oxidative damage analysed (MDA and protein carbonyl derivatives) maintained pre-exercise levels in these cells. This could be related to increases in the expression and/or activities of antioxidant systems. Surprisingly, the increased oxidative capability coexists with a decrease in the antioxidant enzyme activities. This neutrophil response magnifies the importance of other antioxidant defences such as certain vitamins or glutathione [50]. Alternatively, in previous studies we suggested the possibility that neutrophils not only secrete proteins related to inflammation, but rather are capable of secreting antioxidant enzymes as well [19,22]. To confirm this hypothesis we determined the antioxidant enzyme mRNA expression and the CAT localization in neutrophils before and after the cycling stage. All antioxidant enzyme gene expression increased after exercise. In a previous study, we evidenced that the first effect of oxidative stress induced by exercise in lymphocytes is the inactivation of antioxidant enzymes, but after 1.5 h of submaximal exercise the activities of these enzymes are recovered and even increased vs. basal values [51]. A similar pattern of response was observed in neutrophils, although the changes were not significant [51]. After a cycling stage similar to that studied in the present work we found a correlation between the increase in SOD activity and expression in lymphocytes [18]. These previous results evidence that the time gone by from the beginning of exercise to the post-exercise sample taking seems enough for the activation of the enzymes after their synthesis induction.

The increased mRNA contents combined with the lower antioxidant enzyme activities could be explained in two ways. First, the antioxidant enzyme turnover could be increased in a way that their degradation was faster than their synthesis. As result, antioxidant enzyme activities will decrease after exercise. Second, neutrophils could release antioxidant enzymes to extracellular space in order to increase the antioxidant effect surrounding the neutrophil. This secretion could be important to avoid oxidative damage in tissues induced by neutrophil ROS generation. Our immunocytochemistry results are in accordance with the studies of ultrastructural localization of CAT in neutrophils which demonstrate that CAT was localized primarily in the cytoplasm, although in a few number of cells CAT-containing compartments were observed [52]. In our study, we evidenced that pre-exercise CAT labelled with gold is mainly present in the cytoplasm, but also is present in a few vesicles. After exercise the number of marked particles in cytoplasm was reduced and no marked vesicles were observed. Previous studies showed that CAT is colocalized to the specific granules with peroxisomal and lysosomal proteins such as MPO, hydrolases and peroxidases [52]. The presence of CAT into these granules, in addition to the detected increase in extracellular CAT activity after neutrophil activation, agrees with the possible existence of antioxidant enzymes released from neutrophils. However, no mechanism for CAT transport through the plasma membrane has been described. According to several data, the degranulation reaction develops rapidly [8,53]. The stimulus might be related to the action of hormones such as catecholamines or glucocorticoids [54,55]. However, the dynamics of neutrophil protein appearance/disappearance in plasma have been poorly investigated and remain to be elucidated. CAT secretion could be important at local levels in order to avoid oxidative damage induced by neutrophil ROS production.

Recently, it has been proposed that the superoxide anion and mainly its follow-up product hydrogen peroxide, which has a longer lifetime, play a role in cell signalling [56–58], since the production of ROS by NADPH oxidase has been evidenced in a variety of cells other than phagocytes [59,60]. ROS produced by NADPH oxidases are capable to initiate the activation of several transcription factors such as NF-κB participating in the modulation of inflammatory and immune response [16,61] The increased expression of antioxidant enzymes in neutrophil during the acute phase immune response induced by exercise could be mediated by the ROS production. The increase in O_2^- and H_2O_2 that results from the stimulation of membrane-active NADPH oxidase is transient. CAT

secreted by neutrophils could be, at least in part, responsible for ROS returning to basal levels regulating their function as cell signalling intermediates.

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Manuscript IV

Lymphocyte antioxidant response and H_2O_2 production after a swimming session: Gender differences.

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Lymphocyte antioxidant response and H_2O_2 production after a swimming session: Gender differences

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Abstract
This study evaluated the gender differences in response to intense exercise on lymphocyte hydrogen peroxide production, nitric oxide handling and mitochondrial superoxide dismutase (MnSOD) activity and gene expression. Fifteen males and nine females participated voluntarily in the study and performed a swimming session at 75–80% of the maximal capacity. In basal conditions females presented higher lymphocyte MnSOD activity compared to males ($p < 0.05$). Exercise increased MnSOD activity in males ($p < 0.05$) reaching similar values to females. MnSOD gene expression was also increased in males after exercise ($p < 0.05$) but not in females. Nitrite concentration and iNOS gene expression significantly increased only in males after swimming ($p < 0.01$). The exercise decreased UCP-3 gene expression in both genders ($p < 0.05$). Lymphocyte H_2O_2 production significantly increased in males after exercise in non-stimulated and in PMA-stimulated cells ($p < 0.01$). In conclusion, females seem to be more protected against oxidative stress induced by a swimming session. Hydrogen peroxide is mainly produced in males and this subsequently leads to increases in MnSOD gene expression and activity.

Keywords: *Oxidative stress, exercise, gender, antioxidant enzymes, iNOS*

Introduction

During exercise, the oxygen consumption is greatly increased and consequently the reactive oxygen species (ROS) formation is enhanced [1,2]. Major sources of ROS during exercise include leakage of electrons from the mitochondrial electron transport chain and enhanced purine oxidation in muscle, leukocytes activation and increased haemoglobin turnover [3–5]. ROS produced by moderate levels of exercise or regular training programme are capable to induce the expression of antioxidant defences [6,7]. The mechanism by which this regulation is carried out involves transcription factors such as nuclear factor kappa B (NFκB) and activator protein 1 (AP-1) [7,8]. Recently, a wide variety of families of proteins have been reported to be involved in the defence against oxidative stress, as is the case of uncoupling proteins (UCPs) [9]. However, ROS associated with strenuous exercise may react with lipid membranes, proteins and DNA to cause cell damage [10–12].

Inducible nitric oxide synthase (iNOS) is present in many cells involved in immunity and inflammation, which produce high-levels of sustained nitric oxide (NO) synthesis when cells are activated. NO is important as a toxic defence molecule against infectious organisms and also regulates the functional activity, growth and death of many immune cells

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[13]. When NO is generated at high concentrations it is rapidly oxidized to reactive nitrogen oxide species (RNOS) which mediate most of the immunological effects. NO production is increased during exercise [14–16]. NO has vasodilatory effects and increases blood flow, which facilitates the delivery of glucose to the capillary of skeletal muscle.

The lymphocyte antioxidant defences have shown adaptations to oxidative stress to exercise-induced oxidative stress. Increases in vitamin E contents and in antioxidant enzyme activities have been reported after different bouts of exercise [17,18]. It was reported that the expression of the antioxidant enzymes in immune cells is induced and regulated by ROS and cytokines [19]. However, the adaptive response of antioxidant defences did not prevent the oxidative damage in lymphocytes induced after intense exercise [18]. Skeletal muscle generates significant amounts of oxidants during exercise and part of these oxidants are released to extracellular fluid [20]. Repetitive contractions also result in muscle damage, inducing the release of muscle proteins and cytokines into circulation [21]. Both oxidants and cytokines could modulate the lymphocyte response to exercise.

Several studies have focused on the effect of gender on the antioxidant defences and oxidative damage. Mitochondria from females generate less amounts of hydrogen peroxide than those of males and have higher levels of mitochondrial reduced glutathione (GSH) and antioxidant enzymes [22–26]. It has been hypothesized that at least some of these differences could be attributed to the antioxidant properties of female sex hormones [27,28]. Oestrogens also bind to oestrogen receptors and increase the expression of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase [29]. The higher protection in front of oxidative stress might be related to the increased lifespan in females when compared to males [30]. Sex differences in the extent of muscle damage, in the inflammatory response and in the susceptibility to oxidation have been postulated. Generally females have been shown to be more protected than males against both basal and post-exercise oxidative stress [27,31].

In order to elucidate gender differences in response to intense exercise our aim was to study and to compare the effects of a swimming session on lymphocyte hydrogen peroxide production, nitric oxide handling and mitochondrial MnSOD gene expression and activity in males and females.

Materials and methods

Subjects and protocol

Fifteen adolescent males and nine adolescent females participated in this study. They all were swimmers belonging to amateur teams. The characteristics of subjects participating in the study are presented in Table I. Subjects and their parents were informed of the purpose of this study and the possible risks involved before both parents and adolescents gave their written and informed consent to the adolescent taking part in the study. The groups were homogeneous taking into account age, average weight and training sessions. Subjects were non-obese and non-smokers and they were free of any medication. The study protocol was in accordance with the Declaration of Helsinki and was approved by the University of the Balearic Islands bioethics committee and by the ethic committee of investigation of the Govern de les Illes Balears.

The exercise session was performed in an Olympic pool. Swimmers completed a 30 min warm-up prior to starting the exercise protocol consisting of 5 min general movement to warm the body, followed by a brief 10 min stretching session and a 15 min front-crawl swimming at low intensity. After warming the participants started with a series of intermittent 50 m swims of progressively increasing speed for 30 min, with a resting time of $\sim$10–15 s between each swim, reaching a pace corresponding to 75–80% of the maximal capacity of each participant. The correct speed was controlled by means of the time they used to complete each 50 m swim in relation to the best time they achieved in preliminary tests [32]. In these tests participants were asked to complete several bouts of 50 m at their maximum velocity. The time required to complete each 50 m swim was recorded by a trainer using a hand stopwatch and it was used to control the swim speed. These previous tests were also performed to ensure that all the swimmers would be able to complete the exercise protocol.

Experimental procedure

Peripheral venous blood samples were obtained from swimmers in suitable vacutainers with EDTA as anticoagulant before any exercise following an overnight fast and immediately after end the exercise protocol. Lymphocyte fraction was purified from whole blood and quantified in an automatic flow cytometer analyser Techicon H2 (Bayer) VCS

Table I. Anthropometric characteristics of participants.

	Male	Female
Age (years)	16.1 $\pm$ 0.5	14.7 $\pm$ 0.2#
Height (cm)	173 $\pm$ 2	168 $\pm$ 2#
Weight (Kg)	67.8 $\pm$ 3.4	61.0 $\pm$ 1.7
BMI	22.4 $\pm$ 0.9	21.7 $\pm$ 0.6
% Fat body mass	16.0 $\pm$ 1.7	23.1 $\pm$ 1.2#

The values are the mean $\pm$ s.e.m. of 15 males and eight females. # indicates significant differences between genders (Student's *t*-test unpaired data, $p < 0.05$). BMI: body mass index.

system. ROS production was determined in lymphocytes in basal conditions and after activation with PMA. MnSOD activity and the gene expression of UCP-3, MnSOD and iNOS were assessed in lymphocytes. Nitrite levels as marker of NO were also determined in lymphocytes.

Anthropometrical data

Height was determined using a mobile anthropometer (Kawe 44444, France) to the nearest mm, with the subjects head in the Frankfurt plane. Body weight was determined to the nearest 100 g using a digital scale (Tefal, sc 9210, France). Percentage of fat body mass was calculated by bioimpedance with one hand-held BIA unit (Omron®BF 300). Body Mass Index (BMI) was calculated as follows: BMI = weight in Kg/squared height in m).

Lymphocyte purification

Blood samples were processed following an adaptation of the method described by Boyum [33]. Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and was then centrifuged at 900 × g, 18°C for 30 min. The lymphocyte layer was carefully removed. The precipitate containing the erythrocytes and neutrophils was then discarded. The lymphocyte slurry was then washed twice with PBS and centrifuged for 10 min at 1000 × g, 4°C. The cellular precipitate of lymphocytes was lysed with distilled water.

Enzymatic determinations

MnSOD was determined with a Shimadzu UV-2100 spectrophotometer at 37°C. The activity of SOD was estimated using a xanthine/xanthine oxidase system to generate the superoxide anion. This anion produced the reduction of cytochrome c, which was monitored at 550 nm. The superoxide dismutase of the sample removed the superoxide anion and produced an inhibition of the reduction. MnSOD was achieved after specific inhibition of Cu-ZnSOD with 5 mmol/l potassium cyanide [34].

mRNA gene expression

MnSOD, iNOS and UCP-3 gene expression were determined by real time RT-PCR with 18S ribosomal as reference gene. For this purpose, mRNA was isolated from lymphocytes by phenol-chloroform extraction. cDNA was synthesized from 1 μg total RNA using reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. Target cDNAs were amplified in separate tubes using the following procedure: 10 min at 95°C, followed by 40 cycles of amplification. The specific primers and the amplification conditions used for each gene are presented in Table II. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$.

Nitrite levels

Nitrite levels were determined in all lymphocytes by the acidic Griess reaction using a spectrophotometric method. Lysed cells and plasma were deproteinized with acetone and kept overnight at −20°C. Samples were centrifuged for 10 min at 15000 × g at 4°C and supernatants were recovered. A 96-well plate was loaded with the samples or nitrite standard solutions (100 μl) in duplicate; 50 μl sulphanilamide (2% w/v) in 5% HCl was added to each well and 50 μl N-(1-napthyl)-ethylenediamine (0.1% w/v) in water was then added. The absorbance at 540 nm was measured following an incubation of 30 min.

Hydrogen peroxide production

H_2O_2 production in lymphocytes was measured before and after stimulation with phorbol myristate acetate (PMA) using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as indicator. A stock solution of DCFH-DA (1 mg/ml) in ethanol and PMA (1 mg/ml) in DMSO were prepared and stored at −20°C

Table II. Primers and conditions used in Real Time PCRs.

Gene	Primers	Conditions
18S	Fw: **5′-ATGTGAAGTCACTGTGCCAG-3′**	95°C,10 s 60°C, 7 s
	Rv: 5′-GTGTAATCCGTCTCCACAGA-3′	72°C, 12 s
UCP-3	Fw: 5′-CGTGGTGATGTTCATAACCTATG-3′	95°C, 5 s 60°C, 7 s
	Rv: 5′-CGGTGATTCCCGTAACATCTG-3′	72°C, 10 s
MnSOD	Fw: 5′-CGTGCTCCCACACATCAATC-3′	95°C, 10 s 65°C, 5 s
	Rv: 5′-TGAACGTCACCGAGGAGAAG-3′	72°C, 7 s
iNOS	Fw: 5′-TCTGCAGACAGTGCGTTACT-3′	95°C, 10 s 60°C, 10 s
	Rv: 5′-ATGCACAGCTGAGCATTCCA-3′	72°C, 15 s

until analysis. DCFH-DA (30 μg/ml) in PBS was added to a 96-well microplate containing 50 μl lymphocytes suspension. PMA (3 μm) prepared in HBSS or HBSS alone was added to the wells and the fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37°C for 1 h in FLx800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc.).

Statistical analysis

Statistical analysis was carried out using a statistical package (SPSS 14.0 for Windows). Results are expressed as mean ± s.e.m. and $p < 0.05$ was considered statistically significant. The Kolmogorov-Smirnov test was used to evaluate the fit of the data to a normal distribution. Student *t*-test for unpaired data was used to identify differences at baseline regarding the anthropometric characteristics. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analysed were time swimming (S), gender (G) and the interaction of both factors. When significant effects of gender or exercise were found, a student *t*-test for unpaired data was used to determine the differences between the groups involved.

Results

Anthropometric data

The anthropometric characteristics of participants are shown in Table I. Adolescent males and females participating in the study presented similar weight and BMI. However, males were significantly older and taller and had less percentage fat body mass ($p < 0.05$).

The effect of gender and exercise on lymphocyte counts

No significant differences were observed between males and females in the lymphocyte counts in basal conditions and no changes were evidenced after exercise (data not shown).

The effect of gender and exercise on MnSOD

The enzymatic activity and the gene expression of MnSOD in lymphocytes are presented in Figure 1. The basal MnSOD activity (Figure 1A) is significantly higher in females compared to males (43%, $p < 0.05$). Exercise significantly increased the MnSOD activity in males (52% higher, $p < 0.05$) and maintained the basal values in females. The post-exercise activity in males reached to values similar to the females. The MnSOD gene expression (Figure 1B) presented similar basal values in both males and females; however, exercise significantly increased the gene expression in males (59%, $p < 0.05$) but not in females. The post-exercise MnSOD gene expression

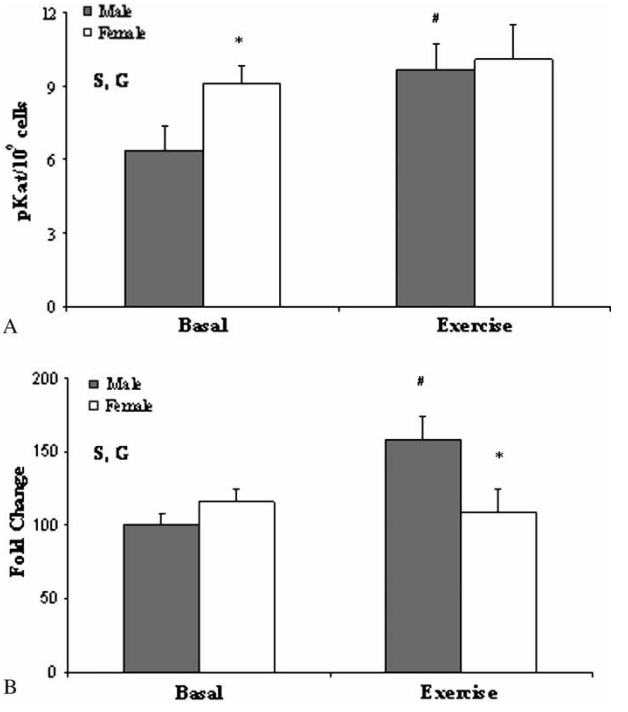

Figure 1. Effects of swimming session and gender on lymphocyte MnSOD activity (A) and MnSOD gene expression (B). The relative quantification of MnSOD expression was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 100. (S): Significant effects of swimming. (G): Significant effects of gender (Two-way ANOVA, $p < 0.05$). *Indicates significant differences between genders. #Indicates significant differences between basal an exercise values. Values are expressed as mean ± s.e.m.

is significantly higher in males when compared with females (50% higher, $p < 0.05$).

The effect of gender and exercise on iNOS

The lymphocyte nitrite levels and the gene expression of iNOS are shown in Figure 2. Both the nitrite levels and the iNOS expression showed the same pattern of change. The basal nitrite levels and iNOS gene expression presented similar values in both males and females. The swimming session significantly increased the nitrite levels and the iNOS gene expression in males (59% and 48%, respectively, $p < 0.01$), but not in females. However, the post-exercise values measured in males were not significantly different compared to the females' post-exercise values.

The effect of gender and exercise on UCP-3 gene expression

The UCP-3 gene expression is presented in Figure 3. Exercise, but not the gender, affected UCP-3 gene expression which decreased about 20% after the swimming session, in both males and females ($p < 0.05$).

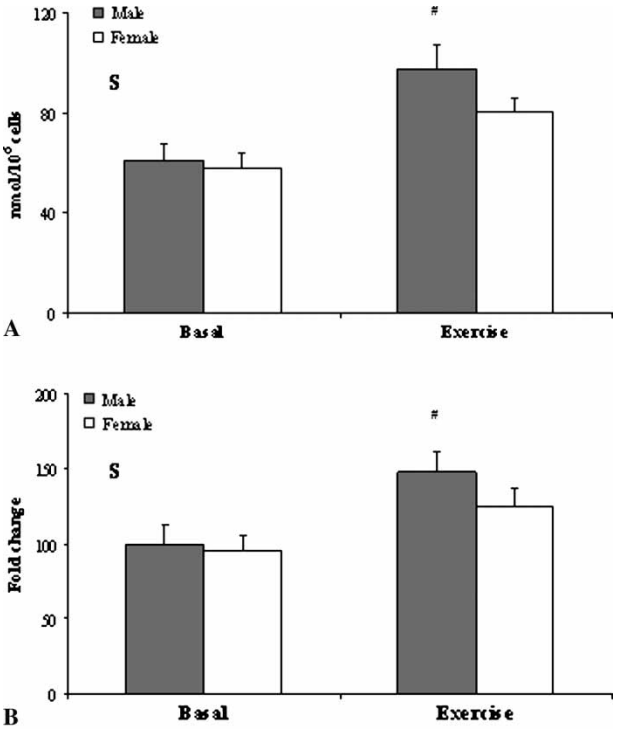

Figure 2. Effects of swimming session and gender on lymphocyte nitrite levels (A) and i NOS gene expression (B). The relative quantification of i NOS expression was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 100. (S): Significant effects of swimming (Two-way ANOVA, $p<0.05$). No significant differences were evidenced between genders. #Indicates significant differences between basal an exercise values. Values are expressed as mean $\pm$ s.e.m.

The effect of gender and exercise on H_2O_2 production

Lymphocyte H_2O_2 production without activation and after activation with PMA are presented in Figure 4. The H_2O_2 production in no-activated lymphocyte showed no significant differences between males and

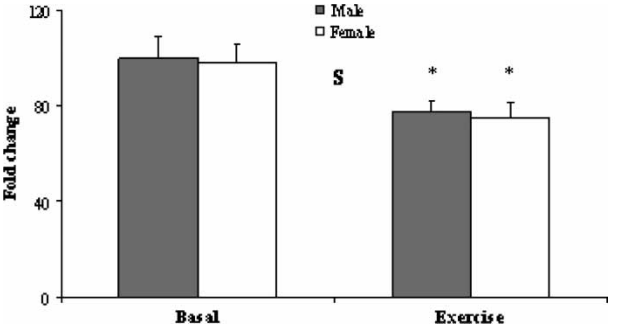

Figure 3. Effects of swimming session and gender on lymphocyte UCP3 gene expression. The relative quantification of UCP3 expression was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 100. (S): Significant effects of swimming (Two-way ANOVA, $p<0.05$). No significant differences were evidenced between genders. #Indicates significant differences between basal an exercise values. Values are expressed as mean $\pm$ s.e.m.

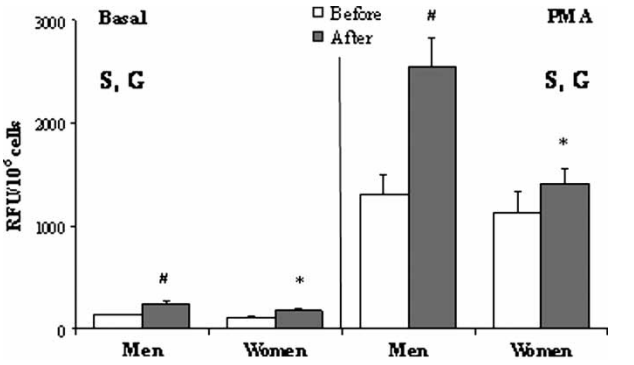

Figure 4. Effects of swimming session and gender on H_2O_2 production in no-activated lymphocytes and in PMA-activated lymphocytes. (S): Significant effects of swimming. (G): Significant effects of gender (Two-way ANOVA, $p<0.05$). *Indicates significant differences between genders. #Indicates significant differences between basal an exercise values. Values are expressed as mean $\pm$ s.e.m.

females in basal conditions. However, after exercise the H_2O_2 production significantly increased only in males (71%, $p<0.01$), being this post-exercise H_2O_2 production significantly higher when compared with the females post-exercise values (25% higher, $p<0.05$). The activation of lymphocytes with PMA increases $\sim$10-fold the H_2O_2 production, but the pattern of response to exercise was similar to the observed in no-activated lymphocytes.

Discussion

Sex differences in muscle damage, in the inflammatory response and in the oxidative stress induced by exercise, have been evidenced by several authors [27,31]. In the present study, we evidenced that the lymphocyte oxidant and antioxidant machinery in females responds less to a swimming training session than in males, which is in agreement with previous results [35]. It has been hypothesized that some of the gender differences could be attributed to the female sex hormones [27]. Therefore, when comparing the basal parameters we only found significant differences between sexes in lymphocyte MnSOD activity, whereas the other parameters were similar in both genders. The higher MnSOD activity in females is in agreement with previous findings [26,36]. It was recently shown that the higher levels of oestrogens in females protect them against ageing by up-regulating the expression of antioxidant genes such as glutathione peroxidase (GPx) and MnSOD via activation the nuclear factor kappa β (NFκβ) [37]. In fact, inhibition of NFκβ signalling pathway prevents this gene induction [37]. Oestrogens also possess phenolic–OH groups which can act as chain-breaking antioxidants in a similar manner to that of vitamin E [5,38]. It was evidenced that the rate of oxidant production by mitochondria from female rats is

significantly lower than from males [26]. Mitochondria from female rats produce approximately half the amount of H_2O_2 generated by male mitochondria, tested in liver and brain [26]. As a result, mitochondria from females suffer less oxidative damage to critical molecules such as mitochondrial DNA or glutathione than those from males [37]. In our study, the ROS production in basal conditions was similar in both genders and this, together with the higher mitochondrial SOD activity in females, suggests that female mitochondria are better protected from oxidative damage in basal conditions.

The physiological response to exercise involves a number of changes in the oxidative balance and in the metabolism of some important biological molecules [39]. Exercise causes an increase in the generation of free radicals by cells [40]. Several authors found that these radicals cause cellular damage only when exercise is exhaustive [41]. However, free radicals not only cause damage but they also have a role in cell signalling [42–44]. The cellular damage is parallel to the increase in the activities of several antioxidant enzymes [45]. In the present study, we studied the lymphocyte capability to produce ROS before and after stimulation with PMA, an agent to activate ROS producing pathways. Lymphocytes from males significantly increased the ROS production after swimming, whereas remained unchanged in females. More than 90% of the oxygen used by cells is consumed in mitochondria being these organelles the main source of ROS [40,46]. Respiratory chain complexes I and III are the main source of mitochondria ROS generation within the cell [47] and their activities are directly correlated with their radical production [48]. Exercise, as result of increased oxygen consumption, induces an increase in mitochondrial oxidant production derived of the increased oxygen utilization. However, any change in mitochondria should be present resulting in an increased ROS production. It was evidenced that the degree of the respiratory chain reduction is inversely related to the rate of electron flow and, consequently, the ROS generation is also affected [49]. As a consequence of this increased ROS production, lymphocytes from males significantly increased the antioxidant enzyme defence to avoid possible cellular damage. The increased ROS after swimming evidenced in males could act as molecular messengers inducing the expression of MnSOD. Recent publications on the production of ROS show that a small increase in H_2O_2 is necessary in the activation of some intracellular signalling pathways, responsible for the development of an adaptive response to exercise-induced oxidative damage [7,50,51]. The ROS activated pathways result in adaptive responses to maintain cellular oxidant–antioxidant homeostasis during exercise.

NO is important for the maintenance of cardiovascular homeostasis and the basal vasodilator tone [52,53]. iNOS is present in many cells involved in immunity and inflammation, which produce high-levels of sustained NO synthesis when cells are activated. iNOS generates greater amounts of NO compared to the constitutive isoforms of NOS. In a previous study, we evidenced that iNOS levels and SOD activity dropped in neutrophils and raised in lymphocytes from male cyclists after exercise [16]. In the present study, we evidenced an increase in iNOS gene expression and nitrite levels—marker of NO—only in males, whereas females maintained basal values. The activation of NFκβ signalling cascade by ROS has been shown to activate the gene expression of iNOS [54]. This response is in accordance with the increased ROS production only evidenced in males. The intracellular accumulation of NO generated by iNOS may produce toxic levels of NO high enough to inhibit key enzymes of the oxidative phosphorylation [55]. *In vitro* experiments documented that NO can attenuate the contractile performance of the skeletal muscle [56,57]. The increased nitrite levels in males after exercise could inhibit any enzyme from the mitochondrial chain facilitating the ROS generation. Both the increased NO and ROS could interact, because the excess of NO production could promote the generation of peroxynitrite in the presence of ROS.

UCP-3 is a member of the mitochondrial uncoupling protein family mainly detected in skeletal muscle but it is not involved in thermogenesis [9]. UCP-3 has been shown to act as an antioxidant by reducing ROS production, but its primary function seems to be extracting fatty acid anions from the mitochondrial matrix, thus protecting mitochondria against the accumulation of fatty acids [58]. In our study, a swimming session induced the down-regulation of UCP-3 expression in lymphocytes from both males and females, which is in contrast with previous works that reported increases in UCP-3 expression in skeletal muscle after an acute bout of exercise [59]. The explanation of these different results could lie in the different kinds of physical activity used or, more probably, in the different cell types analysed. However, UCP-3 gene expression in lymphocytes appears unrelated to differences in radical production between genders.

In conclusion, females seem to have more antioxidant protection than males. An acute bout of exercise as a swimming session induces an activation of the antioxidant machinery only in males to prevent the negative effects of exercise-induced oxidative stress. The lower antioxidant response to exercise observed in female swimmers compared to male swimmers could be explained by the higher oestrogens concentration. These sex differences highlights the importance of studying both genders in exercise

studies rather than generalizing the studies only to males.

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Manuscript V

Antioxidant response and oxidative damage induced by a swimming session: Influence of gender.

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Antioxidant response and oxidative damage induced by a swimming session: Influence of gender

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Abstract
In this study, we examined oxidative stress after a swimming session, the responses of the antioxidant defences, and the influence of gender on these responses. Fifteen boys and eight girls participated voluntarily in the study. Plasma concentrations of 17-β-estradiol, vitamin E, retinol, carotenes, ascorbate, malondialdehyde, and the carbonyl index were determined. Creatine kinase, gamma glutamyl transpeptidase, and lactate dehydrogenase activities, as well as glucose, urea, urate, cholesterol, and triglycerides, were determined in serum. Plasma concentrations of 17-β-estradiol were higher in girls than in boys. Exercise increased plasma ascorbate both in boys and in girls. Malondialdehyde increased in boys but was maintained in girls after exercise. Creatine kinase values corrected for lean body mass were similar in boys and girls at baseline, but the post-exercise values in boys were higher than in girls. A positive correlation was observed in boys, but not in girls, between plasma malondialdehyde and creatine kinase corrected concentrations. Furthermore, a negative correlation was observed between the increase in circulating neutrophils and in creatine kinase activity in girls but not in boys. In conclusion, a swimming session induced higher muscular and oxidative damage in boys than in girls.

Keywords: *Exercise, oxidative stress, gender*

Introduction

Both exhaustive (Ji, 1999; Sjodin, Hellsten Westing, & Apple, 1990) and moderate exercise may increase reactive oxygen species (ROS) production, resulting in oxidative stress (Alessio, 1993; Ji, 1993). Specific sources of ROS during exercise include leakage of electrons from the mitochondrial electron transport chain, xanthine oxidase reaction, haemoglobin oxidation, and activated neutrophils (Ji, 1999; Sjodin *et al.*, 1990). Exercise-induced ROS are also thought to modulate acute-phase inflammatory responses (Cannon & Blumberg, 2000) and to have a role in cell signalling (Jackson, 1999; Reid, Shoji, Moody, & Entman, 1992). Furthermore, strenuous or unaccustomed eccentric exercise results in damage to muscular fibres (Baker, Bailey, Hullin, Young, & Davies, 2004; Clarkson, Nosaka, & Braun, 1992; Suzuki *et al.*, 1999), characterized by prolonged losses in muscle strength and increased concentrations of muscle proteins such as creatine kinase and lactate dehydrogenase in blood (Baker *et al.*, 2004; Clarkson *et al.*, 1992; Kyrolainen, Takala, & Komi, 1998; MacIntyre, Reid, & McKenzie, 1995; Mastaloudis, Traber, Carstensen, & Widrick, 2006; Suzuki *et al.*, 1999). However, we have previously observed that muscular damage is also produced after exercise with an important concentric component, such as a cycling stage (Tauler *et al.*, 2006), a scuba diving session (Ferrer *et al.*, 2007) or a diving session in apnoea (Sureda *et al.*, 2004), the latter two as performed by trained individuals.

The few studies that have investigated the effects of exercise on the redox status of adolescent athletes (Gougoura *et al.*, 2007; Nikolaidis *et al.*, 2007; Ozbay & Dulger, 2002; Santos-Silva *et al.*, 2001) have generally reported increased oxidative stress. There are several metabolic and physiological differences between children and adults at rest and in response to exercise (Cooper, Nemet, & Galassetti, 2004). Children have a greater oxygen cost during exercise (Cooper *et al.*, 2004), which may influence their blood redox status during exercise. Moreover, it has been shown that the changes in intramuscular pH and the ratio of inorganic phosphate to phosphocreatine are smaller during exercise in

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children, indicating that children rely less on anaerobic metabolism than do adults (Cooper *et al.*, 2004). Taken together, these observations suggest that the flow of oxygen to working muscles may be higher in children and, consequently, exercise-induced oxidative stress could be higher in children than in adults (Cooper *et al.*, 2004).

Gender differences in muscle damage, in the inflammatory response, and in the oxidative stress induced by exercise have been reported in humans and animal models (Stupka *et al.*, 2000; Tiidus, 2000). It has been hypothesized that at least some of these differences could be attributed to the female sex hormone 17-β-estradiol (Tiidus, 2000). Some studies have reported significant antioxidant properties for oestrogens, and hence they may be able to influence exercise-induced muscle damage, oxidative stress, and post-exercise inflammation (Tiidus, 2000).

The aim of the study was to compare the oxidative stress and antioxidant defences between trained boys and girls in basal conditions and after an acute bout of exercise – a swimming session.

Materials and methods

Participants and exercise

Twenty-three adolescents (15 boys, 8 girls) volunteered to participate in this study. They were all swimmers on amateur teams. Table I shows the characteristics of the participants. Both the participants and their parents were informed of the purpose of the study and the possible risks involved before the parents gave their written informed consent for the children to take part in the study. The study protocol was in accordance with the Declaration of Helsinki and was approved by the University of the Balearic Islands Bioethics Committee and by the Clinical Investigation Ethics Committee of the Balearic Islands.

Table I. Anthropometric characteristics of participants in the study (mean $\pm$ s_x).

	Boys ($n = 15$)	Girls ($n = 8$)
Age (years)	16.1 $\pm$ 0.5	14.7 $\pm$ 0.2*
Height (m)	1.73 $\pm$ 0.02	1.68 $\pm$ 0.02*
Mass (kg)	67.8 $\pm$ 3.4	61.0 $\pm$ 1.7
BMI (kg $\cdot$ m^{-2})	22.4 $\pm$ 0.9	21.7 $\pm$ 0.6
MUAC (cm)	28.6 $\pm$ 0.8	27.7 $\pm$ 0.5
Triceps skinfold (mm)	7.7 $\pm$ 0.6	14.4 $\pm$ 0.9*
AMA (cm)	26.2 $\pm$ 0.8	22.1 $\pm$ 0.5*
% Fat body mass	16.0 $\pm$ 1.7	23.1 $\pm$ 1.2*
Fat body mass (kg)	11.5 $\pm$ 1.6	14.2 $\pm$ 1.0
Lean body mass (kg)	56.3 $\pm$ 2.2	46.8 $\pm$ 1.2*
Training load (h $\cdot$ day^{-1})	2.32 $\pm$ 0.28	1.60 $\pm$ 0.26

*Significant differences boys vs. girls (Student's *t*-test for unpaired data, $P < 0.05$). BMI = body mass index; MUAC = mid-upper-arm circumference; AMA = arm muscle area.

The exercise session was as follows. Participants in the study warmed up for 30 min before starting the exercise protocol. After the warm-up, the participants began a series of intermittent 50-m swims of progressively increasing speed for 30 min, with 10–15 s rest between swims, reaching a pace corresponding to 75–80% of the maximal capacity of each participant (which was controlled by means of the time they took to complete each 50-m swim in relation to the best time they achieved in the preliminary tests) (Nikolaidis *et al.*, 2007). Next, the participants performed a series of intermittent 50-m swims for 30 min maintaining an intensity of about 75–80% of their maximal capacity. The time required to complete each 50-m swim was recorded by a trainer using a hand-held stopwatch. During the swimming bouts, one of the trainers walked alongside the pool deck giving instructions about pacing. This swimming session supposes a workout of modest intensity for the participants in the study. Tests were performed previously to allow the individual design of the protocol and to ensure that all the swimmers would be able to complete the exercise protocol. In the previous tests, after a controlled warm-up, the participants were asked to complete several bouts over 50 m at their maximum velocity. These tests allowed us to determine the best time for each participant and the individual design of the main exercise session.

Experimental procedures

Venous blood samples were taken from an antecubital vein with suitable vacutainers with ethylenediaminetetraacetic acid (EDTA) as anticoagulant to obtain plasma and without anticoagulant to obtain serum. Venous samples were taken following an overnight fast, before the exercise session, and 1 h after finishing the session. Participants in the study were seated at rest for at least 15 min before blood samples were taken. After the swimming session, the participants were allowed to drink water.

Plasma and serum were obtained after centrifugation (15 min, 1000 *g*, 4°C) of the blood sample. Vitamin E, 17-β-estradiol, retinol, carotenes, ascorbate, malondialdehyde, and protein carbonyl derivative concentrations were determined in plasma. Creatine kinase, gamma glutamyl transpeptidase, and lactate dehydrogenase activities, together with glucose, urea, urate, triglycerides, and cholesterol concentrations, were determined in serum by standard procedures using an autoanalyser (Technicon DAX® System; Parera *et al.*, 1996). The dietary habits of all participants in the study were also determined.

Anthropometric data

The anthropometric variables measured in this study were height, body mass, triceps skinfold thickness, mid-upper-arm circumference, and body composition. Height was determined using a mobile anthropometer (Kawe 44444, France) to the nearest millimetre, with the participant's head in the Frankfurt plane. Body mass was determined to the nearest 100 g using a digital scale (Tefal, sc 9210, France). Participants were weighed barefoot and while wearing light underwear, which was accounted for by subtracting 300 g from the measured weight. The triceps skinfold thickness was measured using a Holtain skinfold caliper (Tanner/Whitehouse, Crymych, UK), and the average of three measurements (right arm) was calculated. Mid-upper-arm circumference, the midpoint between the acromial and olecranon processes, was measured to the nearest 0.1 cm with the participant's right arm relaxed, using a non-stretchable measuring tape (KaWe, 43972, France). Percent body fat and mass of body fat were calculated by bioimpedance using a hand-held BIA unit (Omron®BF 300 body fat monitor). All anthropometric measurements were performed by one observer to avoid inter-observer variation.

Different anthropometric indexes were calculated using these measurements: body mass index [BMI = mass (kg)/squared height (m)]; arm muscle area [AMA = mid-upper-arm circumference (cm) $-\pi$ ⋆ triceps skinfold thickness (cm)]; fat-free body mass as a proxy measure of lean body mass [total body mass (kg) − fat body mass (kg)].

Dietary intake and energy requirements

Dietary habits were assessed using a 3-day dietary record questionnaire completed in the week before the exercise test. A qualified dietician verified and quantified the food records. All food items consumed were transformed into nutrients using a special computerized program based on the European (Feinberg, Favie, & Ireland-Ripert, 1991) and Spanish (Mataix *et al*., 1998; Moreiras, Carbajal, Cabrera, & Cuadrado, 1999) food composition tables. The energy requirements of the participants in the study were calculated according to their basal metabolic rate (BMR) and their physical activity levels (PAL) (FAO/WHO/UNU, 1985, 2001).

Determination of malondialdehyde and protein carbonyl derivatives

Malondialdehyde and protein carbonyl derivatives were analysed in plasma as markers of lipid peroxidation. Malondialdehyde was determined by a colorimetric assay kit (Calbiochem, San Diego, CA, USA). The method used is specific for the determination of malondialdehyde.

Protein carbonyl derivatives were measured in plasma using an adaptation of the method of Levine and colleagues (Levine, Williams, Stadtman, & Shacter, 1994). After deproteinizing the samples with trichloroacetic acid, the precipitates were resuspended with 10 mM 2,4-dinitrophenylhydrazine, and incubated for 60 min at 37°C. Then, the samples were precipitated with 20% trichloroacetic acid, and centrifuged for 10 min at 1000 *g* and 4°C. The precipitate was washed twice with ethanol:ethyl acetate (1:1) to remove free 2,4-dinitrophenylhydrazine. Guanidine (6 M) in 2 mM phosphate buffer 2, pH 2.3 was added to the precipitate, and samples were incubated for 40 min at 37°C. Finally, the samples were centrifuged for 5 min at 3000 *g* at 4°C to clarify the supernatant and the absorbance was measured at 360 nm.

Determination of plasma vitamins and carotenes

Plasma ascorbate was determined by high-performance liquid chromatography (HPLC) with electrochemical detection (Tsao & Salimi, 1982) after deproteinization with meta-phosphoric acid. The mobile phase consisted of 0.05 M sodium phosphate, 0.05 M sodium acetate, 189 μM dodecyltrimethylammonium chloride, and 36.6 μM tetraoctylammonium bromide in 25:75 methanol:H_2O, pH 4.8. The HPLC system was a Shimadzu with a Waters, Inc. electrochemical detector and a Nova Pak, C_{18}, 3.9 × 300 mm column. The potential of the chromatographic detector was set at 0.7 V versus an Ag/AgCl reference electrode.

Vitamin E, retinol, and carotenes were determined in plasma (Tauler, Aguilo, Fuentespina, Tur, & Pons, 2002). The deep-frozen plasma was thawed and mixed to disperse possible precipitates. The extraction of liposoluble vitamins and carotenoids was carried out using *n*-hexane after deproteinization with ethanol containing 0.2% butylated hydroxytoluene. Liposoluble vitamins and carotenes were determined by HPLC in the *n*-hexane extract of plasma after drying under a nitrogen current and redissolving in ethanol. The mobile phase consisted of 550:370:80 acetonitrile:tetrahydrofuran:H_2O. The HPLC was a Shimadzu with a diode array detector and the column was a Nova Pak, C18, 3.9 × 150 mm. α-Tocopherol and retinol were determined at 290 nm and 330 nm respectively. Cryptoxanthin and β-carotene were determined at 460 nm; lutein/zeaxanthine was determined at 450 nm; lycopene was determined at 470 nm.

Determination of 17-β-estradiol

Plasma concentrations of 17-β-estradiol were determined by a direct chemiluminescence immunoassay (Wood, 1984) using an ADVIA Centaur analyser (Siemens M. S. Diagnostics).

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS 10.0 for Windows). Results are expressed as means ± standard errors (s_x). Statistical significance was set at $P < 0.05$. All the data were tested for their normal distribution (Kolmogorov-Smirnov test). Student's *t*-test for unpaired data was used to identify baseline differences in anthropometric characteristics and dietary intake. The effects of gender on the changes induced by the swimming session were tested by a two-way analysis of variance (ANOVA) with gender and the swimming session as factors. When significant effects of gender or exercise were found, a Student's *t*-test for unpaired data was used to determine the differences between the groups involved. We also tested for bivariate correlations (Pearson correlation) between the parameters analysed.

Results

Adolescent boys and girls participating in the study had similar body mass, BMI, and training load (Table I). However, boys were older, taller, and showed significantly greater muscle tissue, both expressed either as kilograms of lean body mass and as arm muscle area.

The dietary intake analysis showed a significant difference between boys and girls in the total energy intake expressed per kilogram of body mass (Table II). The intake was about 38.3% higher in boys than in girls, with higher absolute intakes of fat (45%), carbohydrates (37%), and proteins (58%). Significantly higher intakes of the following nutrients were also found in boys than in girls: thiamine (35%), riboflavin (54%), pantotenic acid (45%), iron (36%), phosphorus (35%), and copper (74%). In adolescent boys, the daily intake of all nutrients analysed was higher than recommended (Moreiras *et al.*, 1999) with the exception of vitamin D. Girls had daily intakes of iron, vitamin D, and folate that were lower than the recommended intakes. No significant differences were observed between boys and girls in the intake of the antioxidants retinol, vitamin C, vitamin E, and carotenes.

Table III shows the effects of the exercise session on plasma antioxidants, oxidative stress markers, and 17-β-estradiol concentrations. A significant effect of gender on retinol concentrations was observed, with higher values in boys than in girls after the swimming session. These differences were related to the intake because plasma baseline retinol concentrations were correlated with retinol intake ($r = 0.556$, $P < 0.001$). Baseline plasma concentrations of vitamins C and E and carotenes were similar in boys and girls. The exercise did not produce any change in the plasma concentrations of carotenes and vitamin E. However, a significant effect of the exercise was observed on plasma ascorbate concentration, with significant increases after the exercise session both in boys (24.8%) and in girls (22.6%). Plasma protein carbonyl derivatives did not change during the study and presented similar baseline values for boys and girls. Plasma malondialdehyde was influenced by both the exercise and gender. The exercise session induced a significant increase in plasma malondialdehyde in boys (23.9%) but girls maintained baseline values. Adolescent boys tended to show higher plasma malondialdehyde concentrations than girls and the difference in malondialdehyde concentration between the genders (36.3%) became significant after the exercise. Baseline plasma malondialdehyde

Table II. Daily energy intake, dietary profile and energy requirements in swimmers participating in the study (mean ± s_x).

	Boys ($n = 15$)	Girls ($n = 8$)
Energy requirements (kcal)	3735 ± 107	2793 ± 43*
Energy intake (kcal)	4049 ± 195	2734 ± 143*
Energy intake (kcal · kg^{-1})	62.5 ± 4.7	45.2 ± 2.8*
Energy intake (kcal · kg LBM^{-1})	73.8 ± 4.9	58.5 ± 2.9*
% Fat	39.4 ± 1.5	38.2 ± 2.4
% Carbohydrates	46.2 ± 1.2	46.2 ± 2.2
% Proteins	15.1 ± 1.6	15.8 ± 0.5
Fat (g · kg^{-1})	2.78 ± 0.27	1.92 ± 0.17*
Carbohydrates (g · kg^{-1})	7.16 ± 0.51	5.22 ± 0.43*
Proteins (g · kg^{-1})	2.79 ± 0.30	1.77 ± 0.10*
Retinol (μg · kg^{-1})	10.9 ± 1.3	6.9 ± 0.9
Vitamin E (mg · kg^{-1})	0.24 ± 0.03	0.29 ± 0.07
Vitamin C (mg · kg^{-1})	2.82 ± 0.50	3.19 ± 0.68
Vitamin D (mg · kg^{-1})	65.7 ± 9.3	56. 7 ± 10.3
Thiamine (mg · kg^{-1})	40.1 ± 2.9	29.6 ± 3.5*
Riboflavin (mg · kg^{-1})	56.9 ± 5.7	36.9 ± 4.7*
Vitamin B_6 (mg · kg^{-1})	65.6 ± 6.5	54.5 ± 10.1
Vitamin B_{12} (μg · kg^{-1})	0.20 ± 0.03	0.14 ± 0.02
Niacin (mg · kg^{-1})	0.54 ± 0.04	0.42 ± 0.04
Pantotenic acid (mg · kg^{-1})	0.16 ± 0.01	0.11 ± 0.01*
Folate (mg · kg^{-1})	7.34 ± 0.67	6.36 ± 1.17
Carotenes (mg · kg^{-1})	45.2 ± 8.3	69.4 ± 14.9
Iron (mg · kg^{-1})	0.34 ± 0.03	0.25 ± 0.03*
Magnesium (mg · kg^{-1})	7.64 ± 0.59	5.99 ± 0.59
Phosphorus (mg · kg^{-1})	36.7 ± 2.6	27.1 ± 1.8*
Calcium (mg · kg^{-1})	23.9 ± 1.8	19.6 ± 2.8
Copper (μg · kg^{-1})	75.0 ± 6.5	42. 9 ± 5.9*
Iodine (mg · kg^{-1})	2.67 ± 0.25	2.47 ± 0.17

Note: Results are expressed per kilogram of body weight. *Significant differences boys vs. girls (Student's *t*-test for unpaired data, $P < 0.05$).

Table III. Changes in plasma antioxidants, oxidative stress markers, and 17-β-estradiol during the swimming session (mean $\pm$ s_x).

	Baseline		Final		ANOVA		
	Boys ($n=15$)	Girls ($n=8$)	Boys ($n=15$)	Girls ($n=8$)	G*E	G	E
Ascorbate (μM)	47.1 ± 3.0	49.6 ± 6.0	$59.8 \pm 4.6^{+}$	$60.8 \pm 5.9^{+}$			*
α-Tocopherol ($\mu g \cdot ml^{-1}$)	8.82 ± 0.28	9.78 ± 0.56	9.46 ± 0.30	9.74 ± 0.58			
Retinol ($\mu g \cdot l^{-1}$)	541 ± 24	481 ± 24	558 ± 24	$475 \pm 26^{\#}$		*	
Lutein/zeaxanthine ($\mu g \cdot l^{-1}$)	41.8 ± 8.4	57.7 ± 10.4	48.3 ± 6.3	52.8 ± 15.3			
Cryptoxanthin ($\mu g \cdot l^{-1}$)	370 ± 66	496 ± 115	380 ± 74	489 ± 108			
Lycopene ($\mu g \cdot l^{-1}$)	338 ± 21	337 ± 51	330 ± 23	322 ± 50			
Carotene ($\mu g \cdot l^{-1}$)	565 ± 85	529 ± 64	552 ± 84	505 ± 68			
MDA (mM)	3.06 ± 0.22	2.34 ± 0.21	$3.79 \pm 0.37^{+}$	$2.78 \pm 0.26^{\#}$		*	*
Carbonyl index (μM)	903 ± 41	901 ± 28	946 ± 44	934 ± 53			
17-β-estradiol (pg/ml)	20.6 ± 2.8	$46.4 \pm 12.1^{\#}$	26.0 ± 2.9	$58.6 \pm 13.3^{\#}$		*	

Note: G, E, and G*E indicate significant effects of the ANOVA factors (two-way ANOVA, $P < 0.05$). G = gender; E = exercise session; G*E gender $\times$ exercise interaction. $^{\#}$Significant difference boys vs. girls. $^{+}$Significant difference before vs. after. MDA = malondialdehyde.

concentrations did not correlate with baseline plasma antioxidant (vitamin C, vitamin E, and carotenes) concentrations. Plasma 17-β-estradiol concentrations were significantly influenced by gender, with significantly higher values in girls than in boys both before (125%) and after (125%) the exercise session. The exercise session did not influence plasma 17-β-estradiol concentrations.

Changes in serum enzyme activities and in circulating numbers of neutrophils are shown in Table IV. Neutrophil number was influenced by both the exercise and gender. Exercise induced significant increases in neutrophils both in boys (159%) and in girls (153%). Boys presented higher numbers of neutrophils than girls, with significant differences between the genders after the exercise (36%). No differences were observed in lactate dehydrogenase activities throughout the study. Serum gamma glutamyl transpeptidase and creatine kinase were significantly influenced by gender, with lower values in girls than in boys. Both baseline (23.7%) and post-exercise (31.2%) gamma glutamyl transpeptidase activities were higher in boys than in girls. Serum creatine kinase activity showed a similar picture, with lower baseline (45.9%) and post-exercise (46.6%) values in girls. As previously suggested (Mastaloudis *et al.*, 2006), creatine kinase and gamma glutamyl transpeptidase serum activities were corrected with lean body mass. After the correction, creatine kinase baseline activities were similar in boys and girls but the observed post-exercise significant difference between the genders was maintained. When the gamma glutamyl transpeptidase values were corrected for lean body mass, the significant effect of the gender factor was maintained, but the observed significant differences between boys and girls after the exercise disappeared.

We tested for correlations between the oxidative and the muscular damage markers as well as the number of neutrophils (results not shown). These correlations were performed to allow us to identify gender differences in the response to the exercise. A significant positive correlation was observed in boys, but not in girls, between plasma malondialdehyde and creatine kinase concentrations, corrected for lean body mass ($r = 0.556$, $P < 0.001$). Furthermore, a significant negative correlation was observed between the increase in circulating neutrophils and the increase in creatine kinase plasma activity in girls ($r = -0.785$, $P < 0.001$) but not boys.

The statistical analysis revealed a significant effect of the two ANOVA factors on changes in serum urate. Both the baseline (22.2%) and the post-exercise (20.4%) values were lower in girls than in boys. The exercise induced an increase in urate that resulted in significantly higher values in boys (25.5%) but not girls. The effect of gender could be attributed to the higher lean body mass of the boys because no differences between the genders were observed in urate concentrations after correction for the participants' lean body mass. The corrected urate values increased significantly in both boys (25%) and girls (29%) after the exercise. No differences were observed in serum urea and glucose concentrations between the genders and during the exercise.

The baseline cholesterol profile and the changes induced by the exercise session are shown in Table V. No significant differences were observed in plasma concentrations of triglycerides, very low-density lipoprotein cholesterol, and low-density lipoprotein cholesterol. However, a significant effect of gender was observed on total and high-density lipoprotein cholesterol. Total cholesterol was higher in girls than in boys, with a significantly higher value after the exercise session (about 13%). This

Table IV. Changes in neutrophil number and in serum parameters during the swimming session (mean ± s_x).

	Baseline		Final		ANOVA		
	Boys ($n=15$)	Girls ($n=8$)	Boys ($n=15$)	Girls ($n=8$)	G*E	G	E
Neutrophils ($10^3 \cdot \mu l^{-1}$)	3.81 ± 0.39	2.87 ± 0.20	9.86 ± 1.18^{+}	7.25 ± 0.79$^{+\#}$		*	*
GGT ($U \cdot l^{-1}$)	13.1 ± 0.7	10.0 ± 0.9	12.9 ± 0.8	8.9 ± 0.6$^{\#}$		*	
GGT/LBM ($U \cdot l^{-1} \cdot kg^{-1}$)	0.23 ± 0.01	0.21 ± 0.02	0.23 ± 0.01	0.19 ± 0.01		*	
LDH ($U \cdot l^{-1}$)	370 ± 12	354 ± 15	408 ± 15	369 ± 16			
CK ($U \cdot l^{-1}$)	327 ± 40	177 ± 29$^{\#}$	429 ± 49	229 ± 37$^{\#}$		*	
CK/LBM ($U \cdot l^{-1} \cdot kg^{-1}$)	6.20 ± 1.00	3.75 ± 0.54	8.10 ± 1.24	4.81 ± 0.68$^{\#}$		*	
Urate ($mg \cdot dl^{-1}$)	5.76 ± 0.29	4.48 ± 0.36$^{\#}$	7.20 ± 0.43^{+}	5.73 ± 0.60$^{\#}$		*	*
Urate/LBM ($mg \cdot l^{-1} \cdot kg^{-1}$)	10.3 ± 0.6	9.4 ± 0.8	12.9 ± 0.8^{+}	12.2 ± 1.2^{+}			*
Urea ($mg \cdot dl^{-1}$)	29.2 ± 1.5	31.0 ± 2.4	35.2 ± 1.9	31.1 ± 2.7			
Glucose ($mg \cdot dl^{-1}$)	84.0 ± 1.6	86.4 ± 2.1	83.6 ± 2.5	84.5 ± 2.1			

Note: G, E, and G*E indicate significant effects of the ANOVA factors (two-way ANOVA, $P < 0.05$). G = gender; E = exercise session; G*E gender × exercise interaction. $^{\#}$Significant difference boys vs. girls. $^{+}$Significant difference before vs. after. GGT = gamma glutamyl transpeptidase; LBM = lean body mass; LDH = lactate dehydrogenase; CK = creatine kinase.

Table V. Serum concentrations of triglycerides, total cholesterol, and lipoprotein-cholesterol during the swimming session (mean ± s_x).

	Baseline		Final		ANOVA		
	Boys ($n=15$)	Girls ($n=8$)	Boys ($n=15$)	Girls ($n=8$)	G*E	G	E
Cholesterol ($mg \cdot dl^{-1}$)	157 ± 7	175 ± 8	155 ± 4	175 ± 8 $^{\#}$		*	
Triglycerides ($mg \cdot dl^{-1}$)	78.8 ± 26.4	53.3 ± 4.1	66.9 ± 6.4	53.9 ± 5.5			
VLDL-cholesterol ($mg \cdot dl^{-1}$)	15.9 ± 5.3	10.8 ± 0.8	16.7 ± 3.5	10.9 ± 1.1			
LDL-cholesterol ($mg \cdot dl^{-1}$)	87.8 ± 6.4	96.3 ± 4.1	86.3 ± 4.7	93.5 ± 4.7			
HDL-cholesterol ($mg \cdot dl^{-1}$)	53.6 ± 2.9	68.1 ± 4.5 $^{\#}$	57.5 ± 3.0	70.4 ± 4.3 $^{\#}$		*	

Note: G, E, and G*E indicate significant effects of the ANOVA factors (two-way ANOVA, $P < 0.05$). G = gender; E = exercise session; G*E gender × exercise interaction. # Significant difference boys vs. girls. VLDL = very low-density lipoprotein; LDL = low-density lipoprotein; HDL = high-density lipoprotein.

difference was due, at least in part, to higher values of high-density lipoprotein cholesterol in girls than in boys both at baseline (27%) and after the exercise (22.4%).

Discussion

As anticipated, the mean energy intake of boys in this study was greater than that of girls. However, when energy intake is expressed per kilogram of body mass and per kilogram of fat-free mass, the difference in energy intake between the genders is reduced (Table II). Energy intake in boys in this study was greater than in girls because they have more lean mass, although after correcting for this factor the difference in energy intake still remains statistically significant. Girls' energy intake values are very similar to the energy requirements proposed for girls of the same age, weight, and physical activity level. Energy intakes observed for both boys and girls in this study match their energy requirements. The differences found between boys and girls in the intake of several nutrients could be attributed to the different total energy intake. In spite of the lower values in girls, an adequate daily nutritional intake was found for almost all the nutrients. Taking into account the fact that the participants in this study were athletes, the most significant deficiency was observed in the girls' iron intake, which was reflected in the lower, but within the normal range, value of plasma ferritin (results not shown).

Swimming is an understudied exercise modality with respect to its effects on blood redox status. To our knowledge, few studies have investigated the effect of acute swimming on both antioxidant and oxidative stress markers (Inal, Akyuz, Turgut, & Getsfrid, 2001; Nikolaidis *et al.*, 2007). These studies showed increased oxidative stress markers such as carbonyl derivatives, lipid peroxidation markers, and oxidized glutathione (Inal *et al.*, 2001; Nikolaidis *et al.*, 2007), but also increased total antioxidant capacity and catalase activity (Nikolaidis *et al.*, 2007). The swimming session performed in the present study was not of a highly stressful nature, and hence the responses observed throughout the study were relatively modest. The oxidative status in boys

and girls during the study was different because the girls showed not only lower plasma malondialdehyde concentrations but also a lower response to the exercise, which is in line with previous results (Dernbach, Sherman, Simonsen, Flowers, & Lamb, 1993). The slight baseline differences observed could be related to differences in training schedule or in antioxidant defences. In fact, the girls showed higher, but non-significant, plasma vitamin E and vitamin C concentrations, which might have induced lower baseline lipid peroxidation as reflected by the lower malondialdehyde levels. This is unlikely because no correlation was found between baseline plasma concentrations of malondialdehyde and antioxidants. The different response to exercise could indicate that girls present a more efficient antioxidant defence than boys because, in terms of intensity and duration, the exercise performed in this study was equivalent in boys and girls. Taken together, these observations indicate that other antioxidants could play an essential role in the prevention of lipid peroxidation. Indeed, it has been reported that oestrogens show an important antioxidant activity (Tiidus, 2000), which might lead to lower peroxidative damage in women. Interestingly, we observed significantly higher 17-β-estradiol concentrations in girls than in boys, which could enhance the antioxidant system in girls, preventing subsequent oxidation. Furthermore, the higher values of serum gamma glutamyl transpeptidase activity in boys than in girls is in line with the higher oxidative stress in boys, because serum gamma glutamyl transpeptidase activity has been suggested as a marker of oxidative stress (Lee, Blomhoff, & Jacobs, 2004). The lack of differences between the genders in previous studies could be related to the age of participants, who were younger than those participating in the present study and presented similar oestrogen concentrations between males and females (Nikolaidis *et al.*, 2007).

The plasma antioxidant response to exercise was also analysed in this study. The observed increase in plasma ascorbate is in agreement with previous findings (Aguilo *et al.*, 2003; Gleeson, Robertson, & Maughan, 1987; Thompson *et al.*, 2001). Regarding the plasma lipophilic antioxidants, it has been reported that their response to exercise is highly dependent on lipoprotein metabolism (Packer, Almada, Rothfuss, & Wilson, 1989). We have shown that the preservation of plasma carotene concentrations is associated with the maintenance of low-density lipoprotein after an exercise session (Aguilo *et al.*, 2005; Tauler *et al.*, 2006), and a correlation between plasma vitamin E and the concentrations of both triglycerides and very low-density lipoprotein cholesterol has been shown by Cases *et al.* (2006). Thus, the lack of changes in plasma vitamin E after the swimming session could be a consequence of the maintenance of very low-density lipoprotein and triglyceride concentrations.

It has been suggested that increases in urate after exercise (Aguilo *et al.*, 2005; Hellsten, 2000; Tauler *et al.*, 2006) are induced by the enhanced muscle energetic metabolism, which increases the degradation of energetic compounds (Hellsten, 2000). When corrected for lean body mass, changes in urate were similar in boys and in girls, suggesting a similar workload and muscle energetic metabolism during the exercise session in boys and in girls. Furthermore, post-exercise urate concentrations have been related to xanthine oxidase activity and ROS production (Gomez-Cabrera *et al.*, 2005; Tauler *et al.*, 2003). The similar values between the genders after exercise could indicate a similar ROS production via xanthine oxidase in boys and girls during the swimming session.

The swimming session induced modest muscular damage as indicated by the non-significant increase in creatine kinase and the maintenance of lactate dehydrogenase. The non-significant increase in creatine kinase could be related to the time of sample, 1 h after finishing the exercise, because several studies have reported maximal creatine kinase increases 24 h post-exercise (Mastaloudis *et al.*, 2006; Stupka *et al.*, 2000; Thompson *et al.*, 2001). In addition to the possible effects of the exercise, creatine kinase was lower at baseline in female than in male adolescent swimmers. These baseline differences could be related to differences between the genders in training load or in antioxidant defences but also to differences in muscle mass. To assess the influence of body muscle content, these serum parameters were corrected for the participants' lean body mass because muscle, which is the main source of these enzymes during exercise, is a major component of lean body mass. After correcting creatine kinase for lean body mass, the results suggest that the significant post-exercise differences between the genders might be related to the higher influence of the exercise session in boys than in girls. In fact, using both expressions, the increase in creatine kinase was significantly higher in boys than in girls. Several studies have demonstrated a higher creatine kinase release from the active muscle in males than in females after exercise (Apple *et al.*, 1987; Dernbach *et al.*, 1993; Janssen *et al.*, 1989; Stupka *et al.*, 2000). In the present study, this difference could be attributed to the higher 17-β-estradiol in girls because of its ability to act as an antioxidant and a membrane stabilizer (Tiidus, 2000) or because of its potential to prevent, at least in part, the inflammatory response. In this sense, it has been reported that similar muscle damage in men and women (characterized by the amount of Z-disk streaming-muscle damage) induced a lower creatine

kinase response in women who, in addition, showed less muscle inflammation (Stupka et al., 2000). Acute inflammation in response to injury is characterized by an increase in circulating neutrophils and their subsequent influx in the damaged skeletal muscle (MacIntyre, Reid, Lyster, & McKenzie, 2000). The neutrophilia observed after the swimming session was higher in boys than in girls, which is in agreement with previously reported results (Miles, Naukam, Hackney, & Clarkson, 1999; Stupka *et al.*, 2000). Furthermore, an inverse correlation was observed between creatine kinase and neutrophil increases in girls but no correlation was found in boys. Taken together, these data suggest a different inflammatory response in males and females, as has been previously suggested (Stupka *et al.*, 2000). Attenuation of muscle neutrophil infiltration has been shown repeatedly in oestrogen supplemented male and ovariectomized female rats after unaccustomed exercise (Stupka & Tiidus, 2001; Tiidus & Bombardier, 1999; Tiidus *et al.*, 2001), suggesting that oestrogen attenuates muscle neutrophil infiltration after exercise. In humans, controversial results have been reported (MacIntyre *et al.*, 2000; Stupka *et al.*, 2000).

The inflammatory response could also influence oxidative damage. It has been suggested that creatine kinase serum activity and malondialdehyde concentrations during exercise could be correlated, indicating that there is a relationship between the free radical attack induced by exercise and the protein leakage to plasma (Sjodin *et al.*, 1990). The positive correlation between plasma malondialdehyde and creatine kinase found in boys but not in girls in this study could indicate that muscular and oxidative damage appear simultaneously in boys. The differences between the genders could be attributed to the higher 17-β-estradiol concentrations in girls because of their antioxidant properties but also, as has been indicated above, because they could decrease the inflammatory response. In fact, these two roles could be related because the inflammatory response includes significant generation of ROS, which can result in an increase in oxidative damage (Cannon & Blumberg, 2000; Reid & Durham, 2002; Suzuki *et al.*, 1999), enhancing the possible protective role of oestrogens during exercise.

In summary, a swimming session induced higher muscular and oxidative damage in boys than in girls. Gender influenced the oxidative stress associated with both exercise and inflammation. Both the inflammatory response and the oxidative damage were prevented, at least in part, in girls. These gender differences could be attributed to the higher concentrations of 17-β-estradiol in girls than in boys.

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Manuscript VI

Antioxidant regulatory mechanisms in neutrophils and lymphocytes after intense exercise.

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Antioxidant regulatory mechanisms in neutrophils and lymphocytes after intense exercise

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Abstract
The aims of this study were to assess the effects of a swimming session on the peripheral blood neutrophil and lymphocyte pro- and antioxidant system, identify any differences between the sexes and the regulatory mechanisms that might induce the immune cell adaptive response to exercise. Twenty-four swimmers (15 males, 9 females) participated in a one-hour swimming session at 75–80% of their maximal capacity. The session induced neutrophilia and decreased antioxidant enzyme activities and ascorbate levels in neutrophils. Malondialdehyde rose in neutrophils in males and females, whereas the carbonyl index only increased in males. Lymphocyte glutathione peroxidase activity was higher in males at baseline and rose as a consequence of exercise. The exercise decreased uncoupling protein-3 and Bcl-2 gene expression. The expression of PPARγ coactivator-1 alpha (PGC-1α) correlated positively with that of sirtuin 3 (SIRT3) and catalase. In summary, a swimming session of one hour at 75–80% of maximal capacity produced oxidative damage in neutrophils and induced the antioxidant defences in lymphocytes. PGC-1α and SIRT3 appear to be key effectors of this adaptive response in lymphocytes. Both the neutrophil and lymphocyte response to exercise were slightly weaker in females than males.

Keywords: *Oxidative, stress, apoptosis, UCP-3, antioxidant enzymes*

Introduction

The damaging effects of reactive oxygen species and free radicals have been widely reported (Ji, 1995; Lodovici, Casalini, Cariaggi, Michelucci, & Dolara, 2000). However, there is growing evidence that low concentrations of reactive oxygen species are able to induce the expression of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase and other defence mechanisms (Vina, Borras, Gomez-Cabrera, & Orr, 2006). The mechanism by which this regulation is carried out is not entirely understood, but it appears to involve transcription factors such as nuclear factor-kappaB (NFκB) (Vina et al., 2006) and co-activators such as peroxisome proliferator-activated receptors gamma coactivator-1 alpha (PGC-1α) (Valle, Alvarez-Barrientos, Arza, Lamas, & Monsalve, 2005). Intense physical activity increases oxygen consumption and the formation of reactive oxygen species (Ji, 1995) and induces an acute phase immune response similar to infection. Oxidative stress associated with exhaustive exercise may impair the immune response (Nieman, 1994), although cells of the immune system do not respond equally. The number of circulating neutrophils rises (McCarthy & Dale, 1988) while their antioxidant enzyme activities tend to decrease (Tauler, Aguilo, Fuentespina, Tur, & Pons, 2002b; Tauler et al., 2003). In contrast, the enzymatic antioxidants of lymphocytes have demonstrated great adaptation to oxidative stress by increasing their activities (Tauler et al., 2004), while the number of circulating lymphocytes decreases after exercise of long duration (McCarthy & Dale, 1988).

Several studies have focused on the effect of gender on antioxidant defences and oxidative damage. Generally, females have been shown to be more protected than males against both basal and post-exercise oxidative stress. It has been reported that females have higher reduced glutathione in whole blood, and higher glutathione peroxidase and superoxide dismutase activities in human erythrocytes (Ho, Chan-Yeung, Chow, Ip, & Mak, 2005) and polymorphonuclear leukocytes

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(Saraymen, Kilic, Yazar, & Cetin, 2003). Increased antioxidant defences in females are accompanied by decreased oxidative damage markers such as plasma malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) as markers of lipid peroxidation (Actis-Goretta, Carrasquedo, & Fraga, 2004) and urinary 8-hydroxydeoxyguanosine as a marker of oxidative DNA damage (Proteggente, England, Rehman, Rice-Evans, & Halliwell, 2002). This higher protection against oxidative stress might be related to the increased lifespan observed in females compared with males, although such studies have not been carried out in humans to date (Vina et al., 2006). An understanding of the immune cells' *in vivo* response to physical activity and the possible differences associated with gender would be useful for planning exercise protocols and dietary guidelines for young healthy people to improve their quality of life.

The aims of this study were to assess the effects of a swimming session on the peripheral blood neutrophil and lymphocyte pro- and antioxidant system, identify any differences between the sexes and the regulatory mechanisms that might induce the immune cell adaptive response to exercise. We hypothesized that a swimming session of one hour at 75–80% of individual maximal capacity would be enough to induce a response in immune cells similar to the acute phase immune response and would induce the activation of the antioxidant defences. We also hypothesized that females would show an enhanced antioxidant status and a more effective antioxidant response to exercise with decreased oxidative damage.

Materials and methods

Participants and protocol

Fifteen boys (age 16.1 ± 0.5 years) and nine girls (age 14.7 ± 0.2 years) volunteered to participate in this study. They were all swimmers on amateur teams. The participants and their parents were informed of the purpose of the study and the possible risks involved before the parents gave their written and informed consent. The study protocol was in accordance with the Declaration of Helsinki and was approved by the University of the Balearic Islands Bioethics Committee.

The participants warmed up for 30 min before starting the exercise protocol. After warming up, the participants started with a series of intermittent 50-m swims of progressively increasing speed for 30 min, with a rest of about 10–15 s between swims, reaching a pace corresponding to 75–80% of individual maximal capacity, which was controlled by means of the time they took to complete each 50-m swim in relation to the best time they achieved in the preliminary tests. The participants then continued the exercise session by performing a series of 50-m swims for 30 min maintaining the intensity at about 75–80% of their maximal capacity. As previously, the swimmers were allowed 10–15 s rest between swims. Pilot tests were performed to allow the individual design of the protocol and to ensure that all the swimmers would be able to complete the exercise protocol. This swimming session supposed a modest intensity workout for the participants. Blood samples were obtained before and 2 h after swimming because changes in antioxidant enzymes and the appearance of oxidative damage are evident 2–3 h after finishing an exercise bout but not immediately after (Ferrer et al., 2007; Sureda et al., 2007).

Experimental procedure

Venous blood samples were obtained from the antecubital vein of swimmers in suitable vacutainers with EDTA as anticoagulant. Lymphocyte and neutrophil cell counts were performed on whole blood in an automatic flow cytometer analyser (Techicon H2 (Bayer) VCS system). Lymphocyte and neutrophil fractions were purified. Catalase, glutathione peroxidase, and superoxide dismutase activities and malondialdehyde, protein carbonyl index, α-tocopherol, and ascorbate were measured in neutrophils and lymphocytes. The expression of catalase, glutathione peroxidase, Bcl-2, sirtuin 3 (SIRT3), and PGC-1α was assessed in lymphocytes. Catalase expression was also measured in neutrophils.

Neutrophil and lymphocyte purification

Blood samples were processed following an adaptation of the method described by Boyum (1964). Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and was then centrifuged at 900 *g*, at 18°C for 30 min. The lymphocyte layer was then carefully removed and the precipitate containing the erythrocytes and neutrophils was incubated at 4°C with 0.15 M ammonium chloride to haemolyse the erythrocytes. The suspension was centrifuged at 750 *g*, at 4°C for 15 min, and the supernatant was then discarded. The neutrophil phase at the bottom was washed first with ammonium chloride and then with phosphate saline buffer, pH 7.4. Finally, the neutrophils were lysed with distilled water.

The lymphocyte slurry was then washed twice with PBS and centrifuged at 1000 *g*, at 4°C for 10 min. The cellular precipitate of lymphocytes was lysed with distilled water.

Enzymatic determinations

All activities were determined in lymphocytes and neutrophils with a Shimadzu UV-2100 spectrophotometer at 37°C immediately after sample collection and cell purification. Catalase (CAT) activity was measured by the spectrophotometric method of Aebi based on the decomposition of H_2O_2 (Aebi, 1984). Glutathione peroxidase (GPx) activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler (1984) using H_2O_2 as substrate. Superoxide dismutase (SOD) activity was measured by an adaptation of the method of McCord and Fridovich (1969). The xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion produced the reduction of cytochrome *c*, which was monitored at 550 nm. The superoxide dismutase in the sample removed the superoxide anion and produced an inhibition of the cytochrome *c* reduction.

Vitamin determinations

Vitamin E was determined in lymphocytes and neutrophils. The cell suspensions were thawed and mixed to disperse possible precipitates. The extraction of liposoluble vitamins and carotenoids was carried out using *n*-hexane after deproteinization with ethanol containing 0.2% butylated hydroxytoluene (BHT). Vitamin E was determined by high-performance liquid chromatography (HPLC) in the *n*-hexane extract after drying under a nitrogen current and re-dissolving in ethanol. The mobile phase consisted of 550:370:80 acetonitrile : tetrahydrofuran : H_2O. The HPLC was a Shimadzu with a diode array detector and the column was a Nova Pak C_{18} (3.9 × 150 mm). Alpha-tocopherol was determined at 290 nm.

Ascorbate was determined in lymphocytes and neutrophils by an HPLC method with electrochemical detection (Tsao & Salimi, 1982) after deproteinization with ortho-phosphoric acid. The mobile phase consisted of 0.05 M sodium phosphate, 0.05 M sodium acetate, 189 μM dodecyltrimethylammonium chloride, and 36.6 μM tetraoctylammonium bromide in 25:75 methanol : H_2O, pH 4.8. The HPLC system was a Shimadzu with a Waters Inc. electrochemical detector and a Nova Pak C_{18} (3.9 × 300 mm) column. The potential of the chromatographic detector was set at 0.7 V versus an Ag/AgCl reference electrode.

Malondialdehyde determination

Malondialdehyde (MDA) as a marker of lipid peroxidation was analysed in lymphocytes and neutrophils by a colorimetric assay kit (Calbiochem, San Diego, CA, USA). The method used is specific for malondialdehyde determination.

Protein carbonyl derivate determination

Protein carbonyl derivates were measured in lymphocytes and neutrophils by an adaptation of the method of Levine (Levine, Williams, Stadtman, & Shacter, 1994) using the precipitates of deproteinized samples. Precipitates were re-suspended with 2,4-dinitrophenylhydrazine (DNPH) 10 mM, and incubated for 60 min at 37°C. Then, the samples were precipitated with 20% trichloroacetic acid, and centrifuged at 1000 *g*, at 4°C for 10 min. The precipitate was washed twice with ethanol : ethyl acetate (1:1) to remove free DNPH. Guanidine 6 M in 2 mM phosphate buffer, pH 2.3, was added to the precipitate, and samples were incubated for 40 min at 37°C. Finally, samples were centrifuged at 3000 *g*, at 4°C for 5 min, to clarify the supernatant and absorbance was measured at 360 nm. The molar absorption of 22,000 $M^{-1} \cdot cm^{-1}$ was used to quantify the protein carbonyl levels. Samples were analysed against a blank of guanidine solution.

mRNA gene expression

mRNA expressions were determined by real-time RT-PCR with 18S ribosomal as the reference gene. For this purpose, mRNA was isolated from lymphocytes and neutrophils by phenol-chloroform extraction. cDNA was synthesized from 1 μg total RNA using reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the Light-Cycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. The primers used are shown in Table I. For all PCRs there was one cycle at 95°C for 10 min, followed by 40 cycles at the conditions shown in Table I.

The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Basal mRNA levels at the beginning of the stage were arbitrarily referred to as 1. The expression of the target gene was normalized with respect to ribosomal 18S.

Statistical analyses

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS version 13.0 for Windows). Results are expressed as means ± standard errors of the mean (s_x). Statistical significance was set at $P < 0.05$. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analysed were swimming and gender. When significant effects were found, a one-way ANOVA was

used to determine the differences between the groups involved. The possible bivariate correlations between the different parameters were also analysed.

Results

The effects of a swimming session and the possible differences associated with gender were studied in neutrophils and lymphocytes. The swimming session induced significant neutrophilia (about 2.5-fold) in both males and females (Table II). Neutrophil counts tended to be higher in males than in females at baseline, and after the session this difference became significant. Lipid and protein oxidative damage markers in neutrophils are also shown in Table II. Malondialdehyde and protein carbonyl derivatives increased after the swimming session. Malondialdehyde rose in both males and females, while the increase in the carbonyl derivative index was only significant in males. No effect of the swimming session or gender was observed in the lymphocyte count, malondialdehyde or the protein carbonyl derivative index (Table II).

The presence of oxidative damage in neutrophils could be a consequence of impaired antioxidant defences. Table III shows the activity and expression of antioxidant enzymes and the antioxidant vitamin concentrations in neutrophils and lymphocytes. The activities of the neutrophil antioxidant enzymes catalase and superoxide dismutase were significantly reduced after the swimming session, and no gender differences were observed. On the other hand, catalase gene expression was increased in neutrophils about two-fold in males but not in females after the swimming session. Ascorbate decreased in neutrophils in both groups as a consequence of the swimming session. An effect of the swimming session and also an interaction between swimming and gender were observed for α-tocopherol in neutrophils. Females showed higher baseline values of α-tocopherol, although the difference was non-significant. The swimming session maintained baseline α-tocopherol values in males while they tended to decrease in females. The antioxidant response induced by exercise was attenuated in lymphocytes when compared with neutrophils. Catalase activity and ascorbate and α-tocopherol concentrations were maintained after the swimming session and no differences were observed between the sexes. In contrast, glutathione peroxidase activity was affected by both swimming and gender. Males showed a higher glutathione peroxidase activity than females at baseline. The swimming session induced an increase in the enzymatic activity in both groups, although it was only significant in males.

The expression of the antioxidant genes glutathione peroxidase, catalase, and SIRT3 in lymphocytes was not affected by the swimming session or gender (Figure 1A, 1B, and 1C, respectively). Exercise affected uncoupling protein-3 (UCP-3) and Bcl-2 gene expression. UCP-3 expression decreased about 20% after the swimming session in

Table I. Primers and conditions used in real-time PCRs.

Gene	Primers	Conditions
18S	Fw: 5′-ATG TGA AGT CAC TGT GCC AG-3′ Rv: 5′-GTG TAA TCC GTC TCC ACA GA-3′	95°C, 10 s 60°C, 7 s 72°C, 12 s
Catalase	Fw: 5′-TTT GGC TAC TTT GAG GTC AC-3′ Rv: 5′-TCC CCA TTT GCA TTA ACC AG-3′	95°C, 10 s 60°C, 10 s 72°C, 15 s
GPx	Fw: 5′-TTC CCG TGC AAC CAG TTT G-3′ Rv: 5′-TTC ACC TCG CAC TTC TCG AA-3′	94°C, 1 s 60°C, 7 s 72°C, 5 s
Bcl-2	Fw: 5′-CTG GTG GGA GCT TGC ATC AC-3′ Rv: 5′-ACA GCC TGC AGC TTT GTT TC-3′	95°C, 5 s 65°C, 5 s 72°C, 5 s
SIRT3	Fw: 5′-GAG CTT CTG GGC TGG ACA GA-3′ Rv: 5′-TGG GAT GTG GAT GTC TCC TAT G-3′	95°C, 10 s 65°C, 5 s 72°C, 7 s
PGC-1α	Fw: 5′-TCA GTC CTC ACT GGT GGA CA-3′ Rv: 5′-TGC TTC GTC GTC AAA AAC AG-3′	95°C, 10 s 60°C, 10 s 72°C, 15 s
UCP-3	Fw: 5′- CGTGGTGATGTTCATAACCTATG-3′ Rv: 5′- CGGTGATTCCCGTAACATCTG-3′	95°C, 5 s 60°C, 7 s 72°C, 10 s

Table II. Effects of a swimming session on neutrophil and lymphocyte counts and oxidative damage (mean $\pm s_x$).

	Before		After		ANOVA		
	Men	Women	Men	Women	S	G	S × G
Neutrophils ($10^6 \cdot ml^{-1}$)	3.81 ± 0.39	2.87 ± 0.20	$9.86 \pm 1.18^{\#}$	$7.25 \pm 0.79^{\#\star}$	×	×	
MDA ($\mu mol \cdot 10^{-9}$ cells)	0.443 ± 0.034	0.438 ± 0.055	$1.11 \pm 0.21^{\#}$	$1.19 \pm 0.27^{\#}$	×		
Carbonyl derivatives ($\mu mol \cdot 10^{-9}$ cells)	3.52 ± 1.06	5.77 ± 1.68	$12.9 \pm 3.6^{\#}$	7.19 ± 1.37	×		
Lymphocytes ($10^6 \cdot ml^{-1}$)	2.21 ± 0.12	2.49 ± 0.25	1.84 ± 0.12	2.16 ± 0.33			
MDA ($\mu mol \cdot 10^{-9}$ cells)	8.30 ± 0.60	10.1 ± 1.4	8.05 ± 0.74	7.84 ± 1.23			
Carbonyl derivatives ($\mu mol \cdot 10^{-9}$ cells)	33.3 ± 2.9	25.7 ± 3.7	31.3 ± 4.4	39.7 ± 3.9			

Note: Two-way ANOVA. S = significant effect of swimming; G = significant effect of gender; S × G = significant interaction between swimming and gender.
$^{\#}$Significant difference before vs. after, $P < 0.05$. *Significant differences men vs. women, $P < 0.05$.

Table III. Effects of a swimming session on the neutrophil and lymphocyte antioxidant system (mean $\pm$ s_x).

	Before		After		ANOVA		
	Men	Women	Men	Women	S	G	S × G
Neutrophils							
Catalase (K · 10^{-9} cells)	13.0 ± 0.8	13.5 ± 1.2	9.91 ± 0.82#	9.60 ± 0.36#	×		
SOD (pkat · 10^{-9} cells)	16.1 ± 2.5	18.9 ± 3.4	10.3 ± 0.9#	11.6 ± 2.4	×		
Catalase expression (fold change)	0.829 ± 0.217	1.31 ± 0.22	1.91 ± 0.41#	1.57 ± 0.37	×		
Ascorbate (mM)	0.98 ± 0.06	1.03 ± 0.07	0.62 ± 0.04#	0.63 ± 0.06#	×		
α-Tocopherol (μM)	87.8 ± 7.0	118 ± 16	91.1 ± 6.5	72.5 ± 4.8	×		×
Lymphocytes							
Catalase (K · 10^{-9} cells)	8.97 ± 0.75	7.99 ± 0.89	9.41 ± 0.76	7.99 ± 1.05			
GPx (nkat · 10^{-9} cells)	91.7 ± 6.3	70.5 ± 3.1	123 ± 11#	100 ± 17	×	×	
Ascorbate (mM)	4.80 ± 0.46	4.47 ± 0.66	4.87 ± 0.50	4.43 ± 0.58			
α-Tocopherol (μM)	494 ± 36	599 ± 67	613 ± 56	587 ± 68			

Note: Two-way ANOVA. S = significant effect of swimming; G = significant effect of gender; S × G = significant interaction between swimming and gender.
#Significant difference before vs. after, $P < 0.05$.

both males and females (Figure 1D), and although we did not find evidence of significant lymphopaenia, Bcl-2 gene expression was also significantly reduced after the session in both groups (Figure 1E). An interaction between swimming and gender was found in the expression of the transcriptional co-activator PGC-1α (Figure 1F). Females recorded a PGC-1α relative expression baseline value about twice that of males, although the difference just failed to reach significance ($P = 0.052$). After the swimming session, PGC-1α expression tended to increase in males whereas it tended to decrease in females.

To better describe the lymphocyte response to a swimming session, we assessed the correlations between the expression of all these genes post-exercise (Table IV). PGC-1α expression was positively correlated with SIRT3, UCP-3, and catalase expression. Bcl-2 mRNA levels were inversely correlated with glutathione peroxidase and positively correlated with UCP-3 expression. SIRT3 and catalase ($P < 0.001$) expression were also positively correlated. Malondialdehyde levels were inversely correlated with Bcl-2 and UCP-3 expression (Table V).

Discussion

The present study examined the neutrophil and lymphocyte pro- and antioxidant response to a swimming session, together with any differences between the sexes. Swimming induced a rise in the number of circulating neutrophils. Neutrophilia after acute long-term exercise is one of the most pronounced effects of physical activity on the immune system (Pedersen & Hoffman-Goetz, 2000; Sureda et al., 2004, 2005; Tauler et al., 2004). The swimming session also decreased the activities of the antioxidant enzymes catalase and superoxide dismutase, as has been observed after a cycling stage (Tauler et al., 2002a) and after a duathlon competition (Tauler et al., 2003). These decreased activities could be attributed to several causes. Antioxidant activities in neutrophils entering the circulation during exercise could be lower than those in the neutrophils present before exercise. On the other hand, exercise could induce a change in enzyme turnover with a decreased enzyme synthesis or increased enzyme degradation. Another possible cause of decreased enzyme activities is a release from neutrophils to plasma. In the present study, the decrease in catalase activity co-existed with a rise in the expression of the enzyme, and we have previously suggested that exercise induces catalase release from neutrophils (Sureda et al., 2007). Neutrophil ascorbate levels were also decreased after the swimming session, in accordance with previous studies (Tauler et al., 2003). The impaired antioxidant defences in neutrophils were accompanied by the appearance of oxidative damage in both lipids and proteins. These results are in contrast with previous studies in which we did not find evidence for oxidative damage in neutrophils after a cycling stage (Sureda et al., 2005) or an apnoea diving session (Sureda et al., 2004). These results together suggest the possibility of a controlled output from neutrophils of both enzymatic and non-enzymatic antioxidants to the extracellular compartment in order to avoid plasma oxidative damage. As a result of this behaviour, the neutrophils become more exposed to oxidation and more susceptible to oxidative damage. A relationship between neutrophil oxidative stress and immune function has been described. Elderly people with lower antioxidant enzyme activities and higher levels

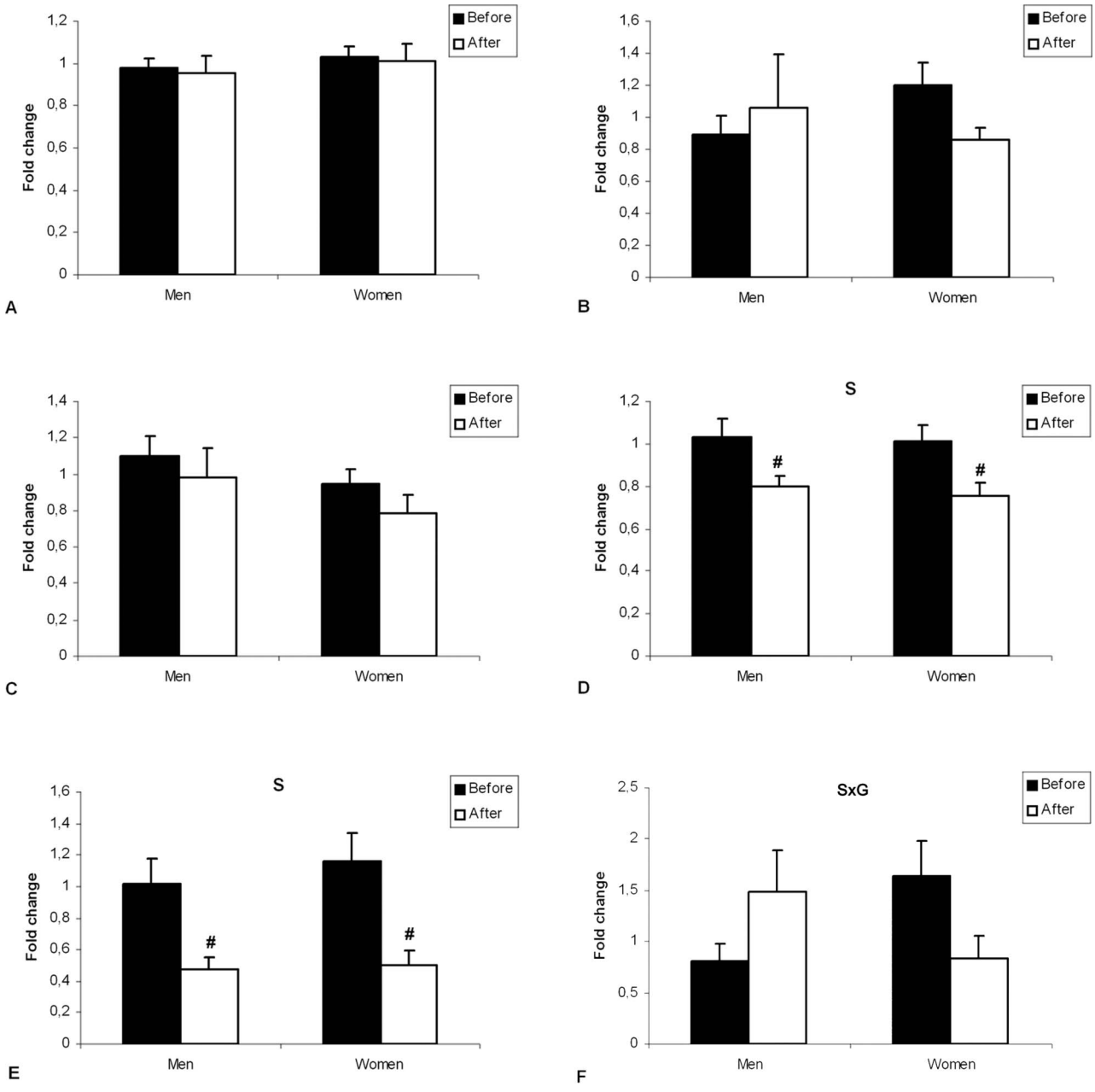

Figure 1. Effects of a one-hour swimming session at 75–80% of maximal capacity on lymphocyte GPx (A), catalase (B), SIRT3 (C), UCP-3 (D), Bcl-2 (E), and PGC-1α (F) gene expression. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. *Note*: Two-way ANOVA. S = significant effect of swimming; G = significant effect of gender; S × G = significant interaction between swimming and gender. #Significant difference before vs. after, $P < 0.05$.

Table IV. Bivariate correlations between the expression of antioxidant genes in lymphocytes after a swimming session.

	PGC1-α	Bcl-2	GPx	SIRT3	Catalase	UCP-3
PGC1-α	1			0.718**	0.762**	0.698**
Bcl-2		1	−0.626*			0.765**
GPx			1			
SIRT3				1	0.853**	
Catalase					1	
UCP-3						1

Notes: *Significant correlation at $P < 0.05$. **Significant correlation at $P < 0.01$.

of oxidative damage markers in neutrophils present lower neutrophil chemotaxis and phagocytosis indexes (De la Fuente, Hernanz, & Vallejo, 2005). Therefore, the decreased antioxidant enzyme activities and the appearance of oxidative damage after the swimming session could induce impairment in neutrophil immune function, thus reducing the effectiveness of the immune defence against pathogenic microorganisms. Non-enzymatic antioxidants such as vitamin C and vitamin E could be useful to balance the production of reactive oxygen species in

the neutrophils primed to the acute phase immune response induced by swimming.

Baseline values of circulating neutrophils did not significantly differ between males and females but after the neutrophilia induced by the swimming session the neutrophil count was higher in males. This difference could be attributable to different intensities of physical activity; however, each individual in both groups carried out the exercise at 75–80% of their own maximal capacity and, consequently, the percentage increase in neutrophil count was similar in both groups (about 250%). Oxidative damage markers and antioxidant enzymes activities were similar in neutrophils between males and females at baseline, although the two groups did not respond equally to exercise. The increase in the carbonyl index as a marker of oxidative modification of proteins and the decrease in superoxide dismutase activity were only significant in males. A significant interaction between gender and exercise was found with respect to α-tocopherol. A decrease in neutrophil α-tocopherol levels was observed in females but not in males, suggesting that this antioxidant vitamin could have been used to prevent the appearance of oxidative damage. Vitamin E supplementation after exercise could be useful to recover intracellular levels of α-tocopherol. Previous studies reported a higher protection of females facing oxidative stress with increased superoxide dismutase activity in neutrophils (Saraymen et al., 2003) or decreased plasma lipid peroxidation markers (Actis-Goretta et al., 2004; Borras et al., 2003; Ilhan, Kamanli, & Ozmerdivenli, 2004).

Lymphocyte counts did not change significantly after the swimming session. The number of circulating lymphocytes has been reported to increase during exercise and fall below baseline values after exercise of long duration (Pedersen & Hoffman-Goetz, 2000; Sureda et al., 2005, 2006). Despite the lack of a significant response in the lymphocyte count, we detected a 50% decrease in the expression of the anti-apoptotic gene Bcl-2 after the swimming session. Bcl-2 is localized in the outer mitochondrial membrane and could be important in the regulation of proton flux in mitochondria, mitochondrial membrane potential ($\Delta\Psi_M$), and the appearance of mitochondrial reactive oxygen species and oxidative damage (Takahashi, Masuda, Sun, Centonze, & Herman, 2004), so it is considered as an antioxidant protein. The results of the present study support the idea that reactive oxygen species, in this case generated by intense exercise, could decrease anti-apoptotic Bcl-2 gene expression, as has been previously reported with Bcl-X_L (Herrera et al., 2001).

UCP-3 is a member of the mitochondrial uncoupling protein family mainly detected in skeletal muscle; it is not involved in thermogenesis (Rousset et al., 2004) but has been shown to act as an antioxidant by reducing the production of reactive oxygen species (Schrauwen & Hesselink, 2004). In the present study, a swimming session surprisingly induced the down-regulation of UCP-3 expression in lymphocytes from both males and females, which is in contrast with previous work that reported increases in UCP-3 expression in skeletal muscle after an acute bout of exercise (Schrauwen & Hesselink, 2003). The explanation for this contradictory behaviour could lie in the different kinds of physical activity used or, more probably, in the different cell types analysed. In the presence of high glucose concentrations, UCP-3 and Bcl-2 expression decreases, $\Delta\Psi_M$ hyperpolarizes, and apoptosis is induced in cultured neuroblastoma cells (Leinninger, Russell, van Golen, Berent, & Feldman, 2004). We evidenced a decrease in both UCP-3 and Bcl-2 expression after intense exercise and a direct correlation between the expression of these two genes after the exercise, thus supporting the idea that both proteins could have related functions in protecting mitochondrial stability by acting as antioxidants.

The antioxidant enzymes studied showed a different response to physical activity. Catalase activity was maintained before and after the swimming session, while that of glutathione peroxidase increased as a result of exercise. Similar results were obtained after a cycling stage (Sureda et al., 2005) and after a submaximal test (Tauler et al., 2004). Together, these results suggest that glutathione peroxidase is the main defence of lymphocytes in the face of oxidative stress. Glutathione peroxidase gene expression was maintained after the swimming session, indicating that up-regulation is at a post-transcriptional level. A gender effect on glutathione peroxidase activity was also detected. The enzyme showed a higher activity in males than in females both before and after swimming. This difference is not due to a different gene expression, so it might be attributable to a different protein concentration or to some post-translational regulatory mechanism. One possible explanation could be that lymphocytes in males respond to exercise by producing more reactive oxygen species and these increased reactive oxygen species could induce greater glutathione peroxidase activity with respect to females, whose reactive oxygen species production would be lower. Several studies have indicated that tissue antioxidant enzyme activities are higher in females than males (Borras et al., 2003; Saraymen et al., 2003), although plasma glutathione peroxidase activity has been found to be higher in males than in females (Ide et al., 2002). When we searched for correlations between antioxidant enzymes and oxidative damage

markers, we found that malondialdehyde was positively correlated with catalase activity and glutathione peroxidase expression and activity, and negatively correlated with Bcl-2 and UCP-3 expression. As we worked with mixed lymphocyte populations, some of the observed changes could be attributable to changes in lymphocyte subset distribution as a result of the new lymphocytes entering the circulation during exercise. However, 2 h after finishing the exercise the lymphocyte count was restored to baseline values. It would appear that the reversion to baseline values subsequent to exercise is partly due to the removal of the same lymphocytes that entered the circulation during exercise (Simpson et al., 2007), so it could be that the observed response is due to the previous circulating lymphocytes rather than to subset variations after exercise. Furthermore, lymphocytes entering the circulation during exercise are then under the same conditions and stimuli, so it could be expected that they respond equally to exercise. In any case, future work should measure the antioxidant response after exercise in specific cell populations obtained after cell enrichment techniques and to compare this response in different lymphocyte subsets.

With these results we show that the events that take place during a swimming session at 75–80% of the maximal capacity, most probably an increase in reactive oxygen species production, up-regulate glutathione peroxidase activity in order to increase lymphocyte protection against oxidative stress. Glutathione peroxidase and catalase activity after exercise are closely related to the accumulation of malondialdehyde, as suggested by the significant correlations. At the same time, Bcl-2 and UCP-3 are down-regulated. The activation of glutathione peroxidase seems to be enough to protect lymphocytes against the potential oxidative stress created by the swimming session because we did not detect significant increases in malondialdehyde or protein carbonyl levels after the exercise and neither did the lymphocyte count fall.

Our next step was to try to gain a better understanding of the molecular mechanisms initiating the antioxidant response in the lymphocytes. PGC-1α enhances the transcription of genes involved in mitochondrial biogenesis and uncoupling (Puigserver & Spiegelman, 2003) and it has recently been reported to co-activate the expression of antioxidant genes such as Mn-superoxide dismutase and catalase, thus improving the antioxidant capacity of cells and preventing oxidative stress mediated apoptosis (Valle et al., 2005). Both chronic and acute exercise have been shown to induce the expression of PGC-1α in muscle cells (Pilegaard, Saltin, & Neufer, 2003; Russell, Hesselink, Lo, & Schrauwen, 2005). However, a swimming session did not directly influence PGC-1α expression in lymphocytes, although an interaction between exercise and gender was observed. Females presented higher baseline values of expression than males, which supports the data for an improved antioxidant status in females. Despite the lack of a significant increase, we found a direct correlation between PGC-1α and catalase mRNA levels after exercise, which is in accordance with previous studies (Shi, Wang, Stieren, & Tong, 2005; Valle et al., 2005). The mechanisms beyond the regulation of PGC-1α in response to exercise are not completely understood. SIRT3, a member of the sirtuin family of histone deacetylases, has been shown to up-regulate PGC-1α expression (Shi et al., 2005). Our study is the first to detect SIRT3 mRNA in human lymphocytes and to analyse the effects of an acute bout of physical activity on SIRT3 expression in lymphocytes. As with catalase and PGC-1α, SIRT3 RNA levels were not affected by the swimming session, but a positive correlation was found between SIRT3 expression and the expression of catalase and PGC-1α after exercise, thus suggesting a close relationship between the expression of these genes in lymphocytes *in vivo*. This suggests that SIRT3 could play an important role in the antioxidant response to exercise in lymphocytes, by activating PGC-1α expression through an unknown pathway, which in turn co-activates the expression of antioxidant enzymes such as catalase and superoxide dismutase. However, some other antioxidant regulatory mechanisms must be involved, since we failed to detect any relationship between glutathione peroxidase expression and PGC-1α or SIRT3 expression.

In conclusion, a one-hour swimming session at 75–80% of maximal capacity was able to induce an acute phase-like immune response and oxidative damage in neutrophils and to induce antioxidant defence in lymphocytes. The antioxidant response in lymphocytes is enough to counteract oxidative damage and apoptosis, and PGC-1α and SIRT3 seem to be key effectors of this adaptive response. Lymphocyte UCP-3 responds to exercise by decreasing its expression. Both neutrophil and lymphocyte responses to exercise were slightly weaker in females than in males.

Table V. Bivariate correlations between oxidative damage and apoptotic markers and antioxidant defences after a swimming session.

MDA vs.	Correlation
Catalase activity	0.660**
GPx activity	0.759**
GPx expression	0.618*
Bcl-2 expression	−0.828**
UCP-3 expression	−0.617*

Notes: *Significant correlation at $P < 0.05$. **Significant correlation at $P < 0.01$.

List of abbreviations

$\Delta\Psi_M$	membrane potential
BHT	butylated hydroxytoluene
CAT	catalase
DNPH	2,4-dinitrophenylhydrazine
GSH	reduced glutathione
GPx	glutathione peroxidase
MDA	malondialdehyde
NFκB	nuclear factor-kappaB
PGC-1α	peroxisome proliferator-activated receptors gamma coactivator-1 alpha
ROS	reactive oxygen species
SIRT3	sirtuin 3
SOD	superoxide dismutase
UCP-3	uncoupling protein-3

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Manuscript VII

Effects of exercise intensity on lymphocyte H_2O_2 production and antioxidant defences in soccer players.

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Effects of exercise intensity on lymphocyte H_2O_2 production and antioxidant defences in soccer players

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Keywords: exercise intensity; lymphocyte; antioxidant enzymes; antioxidant vitamins; oxidative stress.

Abstract

Objective: Physical exercise is capable to enhance or suppress the immune response depending on the intensity and duration of exercise. We studied how exercise intensity

influences the lymphocyte antioxidant response and the induction of cellular oxidative damage.
Design: Eighteen voluntary male pre-professional soccer players participated in this study. Sportsmen played a 60 minutes training match, and were divided in three groups depending on the intensity degree during the match: low, medium and high intensities.
Measurements: Malondialdehyde (MDA), vitamins C and E and heme oxigenase-1 (HO-1) gene expression were measured in lymphocytes. Reactive oxygen species (ROS) production were determined in lymphocytes and neutrophils.
Results: Lymphocyte MDA levels and H_2O_2 production were significantly increased in the group which performed the most intense exercise. Neutrophil counts and ROS production increased progressively with the exercise intensity. Vitamin C significantly decreased after exercise in the highest intensity group respect to initial values, whereas vitamin E levels significantly increased in the medium and high intensity groups. HO-1 gene expression significantly increased in the medium and high intensity groups.
Conclusions: Exercise intensity affects the lymphocyte and neutrophil oxidant/antioxidant balance, but only exercise of high intensity induces lymphocyte oxidative damage.

Introduction

The physiological response to the physical exercise involves a number of changes in the oxidative balance and in the metabolism of some important biological molecules[1]. Physical exercise is characterised by an increase in oxygen consumption by the whole body and by an increase in reactive oxygen species (ROS) production[2]. The main sources of ROS during exercise are the mitochondrial respiratory chain, xanthine oxidase catalyzed reaction and neutrophil activation[3]. ROS are known to cause oxidative modifications of lipids, proteins and nucleic acids leading to cell and tissue damage[4-7]. Under physiological conditions, these deleterious species are mostly removed by the cellular antioxidant systems. Exercise-induced ROS are also thought to modulate acute-phase inflammatory responses[8] and to have a role in cell signaling inducing specific cellular adaptations to exercise[9 10].

Exhaustive exercise elicits a stress response similar to the acute phase immune response[8]. Exercise affects lymphocytes as reflected in total blood cell counts and lymphocyte proliferative response[11 12]. It is widely accepted that athletes undergoing intensive training and competition schedules are at increased risk of developing upper respiratory tract infections[13]. Strenuous physical exercise, characterised by a remarkable increase in oxygen consumption with concomitant ROS production, could lead a oxidative stress situation and cell damage[3]. It has been evidenced that athletes undergoing regular and adequate training show improved intracellular antioxidant status[14 15] and increased resistance to upper respiratory tract infections[16].

Soccer is one of the most popular sports worldwide, but just a few studies have focused on the effect of soccer practice in the antioxidant status of players. Competitive soccer engages many of the body's systems to a major extent to a point where can appear oxidative-derived damage[17]. Some studies have compared the oxidant and antioxidant status of soccer players and sedentary controls. Total antioxidant capacity, superoxide dismutase activity, uric acid, ascorbic acid and tocopherol plasma levels were all higher in soccer players, while MDA levels were lower[14 18]. The levels of

autoantibodies against oxidized LDL in professional soccer players are increased as result of intensive training-induced oxidative stress[19]. Despite of these studies, there are few data concerning the effects of a soccer match in the antioxidant status of players.

The aim of this study was to determine the differential effects of the exercise intensity on the antioxidant response and on the cellular oxidative damage in lymphocytes induced by a training soccer match. Neutrophil capability to produce ROS was also determined for comparison with lymphocytes.

Materials and methods

Subjects and protocol

Eighteen voluntary male pre-professional soccer players participated in this study after giving their written consent to participate. The protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of CAR-Sant Cugat (Barcelona). All sportsmen participating in the study had a controlled diet and expended similar periods of training and competition.

The sportsmen played a training soccer match of 60 minutes. The exercise intensity was determined by a pulsometer, and sportsmen were divided in three groups (n=6) depending on intensity degree. As the cardiac heart rate increases linearly to the oxygen consumption[20] we can indirectly evaluate the work done during a maximal and intervallic exercise through the heart rate[21]. We categorize the subjects under the perspective of the work performed during the training sessions and the competition in relation to the reference values of a progressive and maximal exercise test. Five metabolic zones are usually considered. From zone one (Z1) to zone five (Z5), the relation to the maximal oxygen consumption are respectively <70%, 70-80%, 80-90%, 90-100% and, 100% or higher. Groups were simplified to three (Table 1), the Low intensity (more than 50% of match into the zones Z1 plus Z2) the Medium intensity (more than 50% of match into the zone Z3) and the High Intensity (more than 50% of the match into the zones Z4 plus Z5).

Table 1. Distribution of groups

	Z1-Z2	**Z3**	**Z4-Z5**
Low Intensity (%)	61.2 ± 5.7	36.5 ± 5.2	2.32 ± 1.16
Medium Intensity (%)	26.0 ± 3.7	62.8 ± 3.8	11.1 ± 3.2
High Intensity (%)	2.70 ± 0.63	21.7 ± 4.3	75.6 ± 4.8

Percentage of time which the players performed low, medium or high exercise intensity. From zone one (Z1) to zone five (Z5), the relation to the maximal oxygen consumption are respectively <70%,70-80%, 80-90%,90-100% and, 100% or higher. Groups were simplified to three, the Low intensity (Z1 plus Z2 more than 50% of the total time) the Medium intensity (Z3 more than 50% of the total time) and the High Intensity (Z4 plus Z5 more than 50% of the total time).

The three groups had similar anthropometric values (Table 2).

Table 2. Anthropometric and physiological parameters

	Low intensity	Medium intensity	High intensity
Age (years)	20.2 ± 0.4	19.8 ± 0.3	19.7 ± 0.4
Height (cm)	179 ± 2	176 ± 2	175 ± 3
Weight (Kg)	76.1 ± 1.8	72.0 ± 1.9	75.5 ± 2.4
BMI	23.6 ± 0.5	23.0 ± 0.7	24.8 ± 0.9
VO2 max (ml·kg-1·min-1)	56.6 ± 0.8	58.4 ± 2.4	56.2 ± 1.5

One-way ANOVA, no significant differences were evidenced between groups. Each value represents mean ± s.e.m, n=6.

Venous blood samples were obtained from the antecubital vein with EDTA as anticoagulant. Samples were obtained in basal conditions and immediately after finishing the exercise. Blood samples were used to purify lymphocytes and neutrophils, and to obtain plasma. Lymphocyte and neutrophil counts were quantified in an automatic flow cytometer analyser Techicon H2 (Bayer®) VCS system.

Lymphocyte, neutrophil and plasma purification

Blood cells were immediately purified from whole blood following an adaptation of the method of Boyum[22]. Blood was introduced on Ficoll and centrifuged at 900xg, at 4°C for 30 min. The lymphocyte layer was carefully removed and washed twice with PBS and centrifuged for 10 min at 1,000xg, 4°C. This method ensures that 95±5% of cells in fraction are mononucleocytes with 95±5% viability. The cellular precipitate of lymphocytes was lysed with distilled water. The precipitate obtained after centrifugation with Ficoll, containing erythrocytes and neutrophils was incubated at 4ºC with ammonium chloride 0.15 M to hemolyse erythrocytes. The suspension was centrifuged at 750xg, at 4ºC for 15 min and the supernatant was discarded. The neutrophil phase at the bottom was washed first with ammonium chloride and then with PBS. Neutrophils were resuspended in Hank's balanced salt solution (HBSS) for chemiluminescence assays.

Plasma was obtained after centrifugation for 15 min at 1,000xg at 4°C of another blood sample and was stored at -80°C until use.

Lymphocte MDA concentration

MDA as a marker of lipid peroxidation was analyzed in lymphocytes by a colorimetric assay kit (Calbiochem, San Diego, CA, USA) by following manufacturer's instructions. This assay kit is specific for MDA, avoiding the poor reproducibility and the interference by several agents of the classical determination by the TBARs method[23].

Lymphocyte Vitamin C and vitamin E determination

Samples for vitamin C were deproteinized with 5% meta-phosporic acid, centrifuged for 5 min at 15,000xg at 4ºC, and supernatants recovered. The mobile phase consisted of 0.05 M sodium phosphate, 0.05 M sodium acetate, 189 µM dodecyltrimethylammonium chloride and 36.6 mM tetraoctylammonium bromide in 25/75 methanol/water (v/v), pH 4.8. The HPLC system was a Shimadzu with a Waters Inc electrochemical detector and Nova Pak, C18, 3.9 x 150 mm column. The potential of the chromatographic detection was set at 0.7 V versus an Ag/AgCl reference electrode. Vitamin C was quantified by using a standard curve of known concentration.

Vitamin E was extracted from lymphocyte lysates using n-hexane after deproteinization with ethanol. Vitamin E concentration was determined by HPLC in the n-hexane extract after drying the samples under nitrogen and dissolving in methanol. The mobile phase consisted of 550:370:80 acetonitrile:tetrahydrofuran:H_2O, and the column was a Nova Pak, C18, 3.9 x 150 mm. α-tocopherol isoform was determined at 290 nm and was quantified by comparison to a standard curve of known concentration.

Vitamin concentrations were calculated by taking into account the lymphocyte volume of $21 \cdot 10^{-6}$ µl/lymphocyte[24].

Lymphocyte hydrogen peroxide production

H_2O_2 production in lymphocytes was measured before and after stimulation with phorbol myristate acetate (PMA) using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as indicator. DCFH-DA (30 µg/ml) in PBS was added to a 96-well microplate containing lymphocyte suspension. PMA (3 µM) prepared in HBSS or HBSS alone was added to the wells and the fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37ºC for 1 hour in FLx800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc., U.S.A.).

Neutrophil Chemiluminescence assay

Opsonized zymosan (OZ) was used as neutrophil stimulant. Zymosan A (Sigma) was suspended in HBSS at a concentration of 1 mg/ml and incubated with 10% human serum at 37ºC for 30 min, followed by centrifugation at 750xg for 10 min at 4ºC. The precipitate was washed twice in HBSS and finally resuspended in HBSS at 1 mg/ml. OZ suspension (100 µl) was added to a 96-well microplate containing 50 µl neutrophil suspension and 50 µl luminol solution (2 mM in PBS, pH 7.4). Chemiluminescence was measured at 37ºC for 90 min in FLx800 Microplate Flurescence Reader.

Real-time reverse transcriptase-polymerase chain reaction

Lymphocyte mRNA was isolated by phenol-chloroform extraction. cDNA was synthesized from 1 µg total RNA using reverse transcripase with oligo-dT primers. Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. The primers used were: HO-1, forward: 5'-CCAGCG GGCCAGCAACAAAGTGC-3', reverse: 5'-AAGCCTTCAGTGCCCACGGTAAGG-3' and 18S, forward: 5'-ATGTGAAGTCACTGTGCCAG-3', reverse: 5'-GTGTAATCCGTCTCCACAGA-3'. The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of amplification at 95 °C for 0 s, 60 ºC for 5 s and 72 ºC for 10 s for HO-1 and for ribosomal 18S 40 cycles at 95 ºC for 10 s, 60 ºC for 7 s and 72 ºC for 12 s. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Basal mRNA levels of the low intensity group were arbitrarily referred to as 1. The expression of target gene was normalized with respect to ribosomal 18S.

Statistical analysis

Statistical analysis was carried out using a statistical package (SPSS 12.0 for Windows). Results are expressed as mean ± s.e.m. and $p<0.05$ was considered statistically significant. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analyzed were the exercise intensity (Fc) and the soccer match (E). When significant effects were found, a one-way ANOVA was used to determine the differences between the groups involved.

Results

Lymphocyte number and hematocrit were similar in the three groups in basal conditions and did not change during the exercise in any of the three studied groups (data not shown).

No significant differences were observed between groups in the lymphocytes basal ROS production (Figure 1). ROS production in non-activated lymphocytes significantly increased only in the most intense group after the match (24%). This increase is significantly different when compared with the post-exercise values obtained in the low intensity group. In PMA-activated lymphocytes, ROS production maintained initial values in the groups which performed the lower and medium intensity exercise. ROS production significantly increased in the high intensity group after exercise (33%), being the final values in this group significantly higher respect to the final values measured in the low and medium intensity groups.

Neutrophil number and the ROS production were similar in the three groups in basal conditions (Table 3).

Table 3. Neutrophil counts and ROS production

	Low intensity	Medium intensity	High intensity
Neutrophils (10^6/ml)			
Before	3.26 ± 0.21	3.16 ± 0.23	3.37 ± 0.22
After	4.41 ± 0.44*	5.06 ± 0.43* &	6.17 ± 0.51* #
RLU/min/10^6 cells			
Before	1887 ± 120	1908 ± 116	1912 ± 126
After	1765 ± 103	2208 ± 292	2680 ± 182* &

Two-way ANOVA, $P<0.05$. No significant differences were evidenced between basal values. * before v. after. & differences respect to low post-exercise group. # differences respect to medium post-exercise group. Each value represents mean ± s.e.m, n=6.

Exercise induced an increase in the number of circulating neutrophils, and the increase degree depends on the exercise-intensity. In the low intensity group circulating neutrophils increased 36%, in the medium group 61% and in the high group 83%. ROS production in zymosan stimulated neutrophil only increased significantly in the high intensity group respect to pre-exercise values. This increase is significant different compared to post-exercise values of the low-intensity group.

Both exercise and exercise intensity influenced lymphocyte vitamin C and E levels (Table 4).

Table 4. Lymphocyte vitamins C and E concentration and MDA levels

	Low intensity	Medium intensity	High intensity
Vitamin C (mM)			
Before	3.28 ± 0.32	3.41 ± 0.49	3.39 ± 0.51
After	3.24 ± 0.18	2.71 ± 0.20	2.23 ± 0.10* &
Vitamin E (mM)			
Before	0.49 ± 0.04	0.52 ± 0.04	0.50 ± 0.03
After	0.53 ± 0.08	0.63 ± 0.03*	0.67 ± 0.01* &
MDA (nmol/10^6cells)			
Before	3.44 ± 0.27	3.80 ± 0.39	3.75 ± 0.14
After	4.31 ± 0.23	4.58 ± 0.11	5.33 ± 0.16*

Two-way ANOVA, $P<0.05$. No significant differences were evidenced between basal values. * before v. after; & differences respect to low post-exercise group. Each value represents mean ± s.e.m, n=6.

Vitamin C significantly decreased after exercise in the highest intensity group respect to initial values (34%), whereas maintained basal values in the other groups. This vitamin C decrease was significantly different when compared to the vitamin C levels obtained in the lower intensity group, measured after finishing the match. Vitamin E levels significantly increased in the medium (28%) and high (34%) intensity groups after exercise. The after-match values in the most intense group were significantly higher than the ones obtained in the low intensity group after the match. There were no significant differences in the basal values of MDA concentration between groups. MDA levels were significantly increased in the high intensity group after the match (42%), whereas the other two groups maintained initial values. The increase in MDA levels in the high intensity group after the match was not significantly different respect the results obtained in the medium and low intensity groups.

HO-1 expression was similar in the three groups in the basal point (Figure 2). After the training match, HO-1 expression significantly increased in the groups which performed the medium and high exercise intensities (57% and 86% respectively). However, the post-exercise values in the medium and high intensity groups were not significantly different respect the post-exercise values measured in the low intensity group.

Discussion

Moderate exercise is a healthy practice; however, exhaustive exercise induces oxidative stress. Moderate exercise attenuates lymphocyte apoptosis induced by oxidative stress, possibly by improving intracellular anti-oxidative capacity[15]. In a previous studies, we reported that a mountain cycling stage induced a significant lymphopenia and high levels of oxidative stress in lymphocytes[25] whereas a cycling stage without mountainous terrain maintained lymphocyte counts[26]. These results suggest that the exercise intensity could be the responsible of the stress-induced changes. In fact, in the present study, MDA concentration increased only in the group that performed the most intense. We also reported an inverse correlation between lymphocyte protein carbonyl derivates and lymphocyte number[11].

We studied the lymphocyte capability to produce ROS by using DCFH-DA before and after stimulation with PMA as possible source of oxidative stress. However, DCFH can suffer an auto-oxidation that appears to form trace amounts of H_2O_2, but the rate of auto-oxidation should be of equal importance in each sample[27]. The progressive increase in the capabilities of ROS production in the groups which performed the moderate (with no significant differences) and high intensities after the match indicates a relationship between exercise intensity and the appearance of oxidative stress. Exercise probably induces an increase in mitochondrial oxidant production as result of the increased oxygen availability. However, recent publications on the production of ROS show that a small increase in H_2O_2 is necessary in the activation of some intracellular signalling pathways, responsible for the development of an adaptive response to exercise-induced oxidative damage[28 29].

Exercise induces an increase in the number of circulating neutrophils related to the intensity of the physical activity. In a previous studies, we evidenced that an exhaustive exercise such as a duathlon competition or a cycling mountain stage increases about 4-fold the neutrophil counts[30 31], while in another study, a flat cycling

stage neutrophils number only increases 2-fold[32]. Circulating neutrophils of the high intensity group but not in the low and medium intensities seem primed for oxidative burst after exercise as is evidenced by the progressive increase in the maximum luminol chemiluminescence. This response is similar to the obtained for lymphocytes. The increased pre-activation state of neutrophils from the most intense exercised group could contribute, at least in part, to the instauration of oxidative stress

Deficiency of antioxidant nutrients appears to hamper antioxidant systems and augment exercise-induced oxidative stress and tissue damage[3 33]. Vitamin C prevents initiation of lipid peroxidation and spares other critical antioxidants including α-tocopherol and urate[34]. Vitamin C is also an essential metabolite for cell and tissue metabolism, especially for collagen synthesis and for regulation of HIF-1α[35 36]. Although vitamin C may interact with 'free' active metal ions contributing to oxidative damage[37], its relevance *in vivo* has been a matter of controversy. Lymphocyte vitamin C levels decreased significantly only in the group which performed the most intense exercise, probably because this group produced more ROS and consequently more vitamin C was consumed. Vitamin E levels were unchanged in the low intensity group, but significantly increased in the other two groups. These results are in agreement with the increase observed in lymphocyte vitamin E just after a half marathon or after a mountain cycling stage[28 38]. It seems that vitamin E lymphocyte uptake is activated by oxidative stress in order to protect the cell from the action of ROS, according to the levels of oxidative stress.

When analysed the HO-1 gene expression a significant increase was evidenced in the medium and higher intensity groups. HO-1 is an antioxidant stress protein induced by stressful and inflammatory stimuli[39]. HO-1 is particularly sensitive to acute exercise being activated by the NF-kB pathway. Several authors have evidenced an increase in HO-1 after a half-marathon[39] or after a treadmill test until exhaustion,[40] but not after a short run or eccentric exercise[39]. Our results suggest that HO-1 is activated depending on the degree of ROS production

In conclusion, exercise affects the lymphocyte antioxidant system response and induces cellular oxidative damage depending on its intensity. Intense exercise enhances lymphocyte and neutrophil ROS production, produces lipid peroxidative damage and lymphocyte ascorbate consumption, activating the antioxidative response as evidenced by the increase in lymphocyte HO-1 expression. Intense exercise-induced oxidative stress is probably related to a disturbance in the redox homeostasis. Thus, physical exercise provides an excellent model to study the relationship between pro-oxidants and the antioxidant defenses in healthy subjects.

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Figure legends

Figure 1. Lymphocyte ROS production. Lymphocyte ROS production without stimulation (A) and stimulated with PMA (B). Two-way ANOVA, $P<0.05$. No significant differences were evidenced between basal values. * before v. after; # differences respect to low and medium post-exercise groups; & differences respect to low post-exercise group. Each value represents mean ± s.e.m, n=6.

Figure 2. Lymphocyte HO-1 gene expression. Two-way ANOVA, $P<0.05$. No significant differences were evidenced between basal values. * before v. after. Basal mRNA levels of the low intensity group at the beginning of the stage were arbitrarily referred to as 1. Each value represents mean ± s.e.m, n=6.

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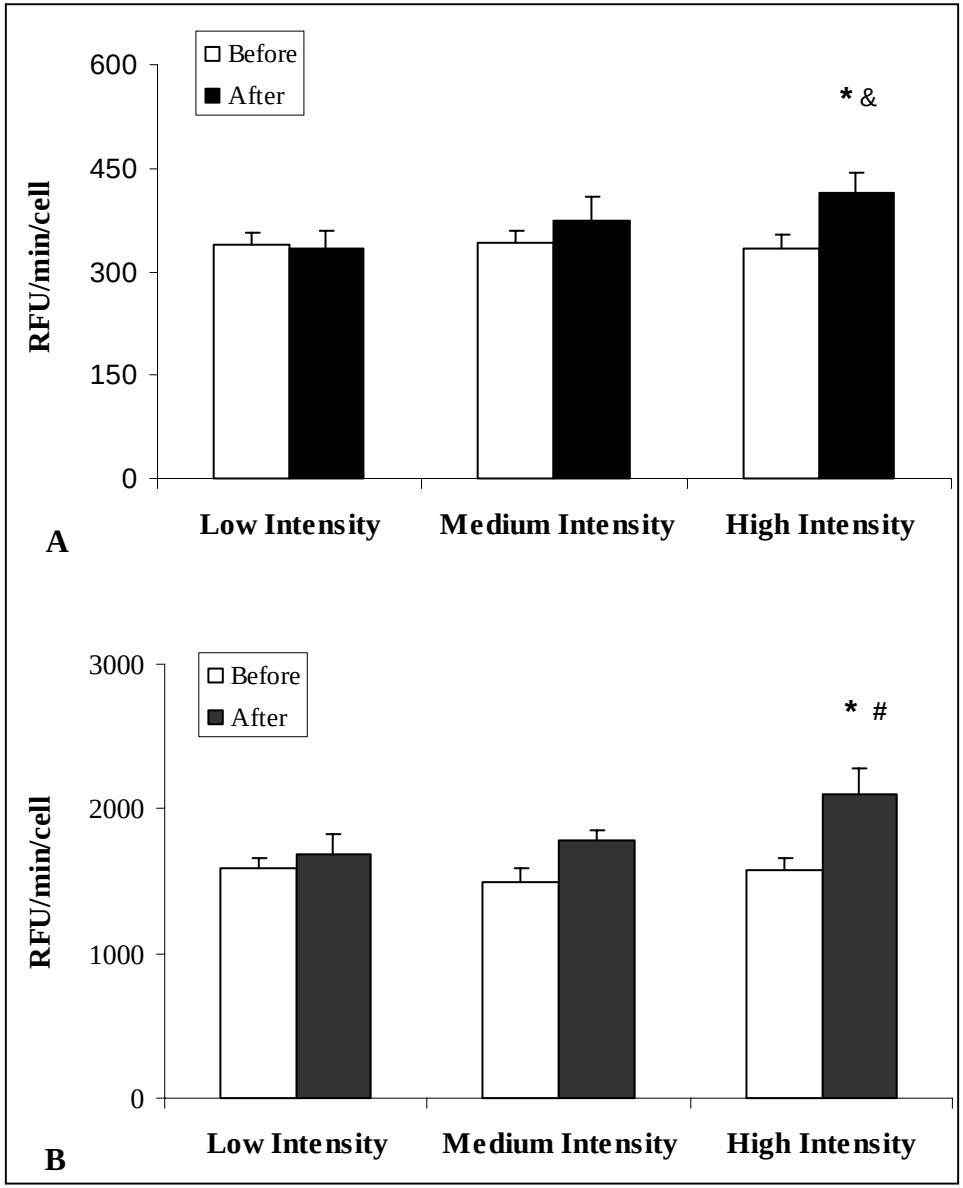
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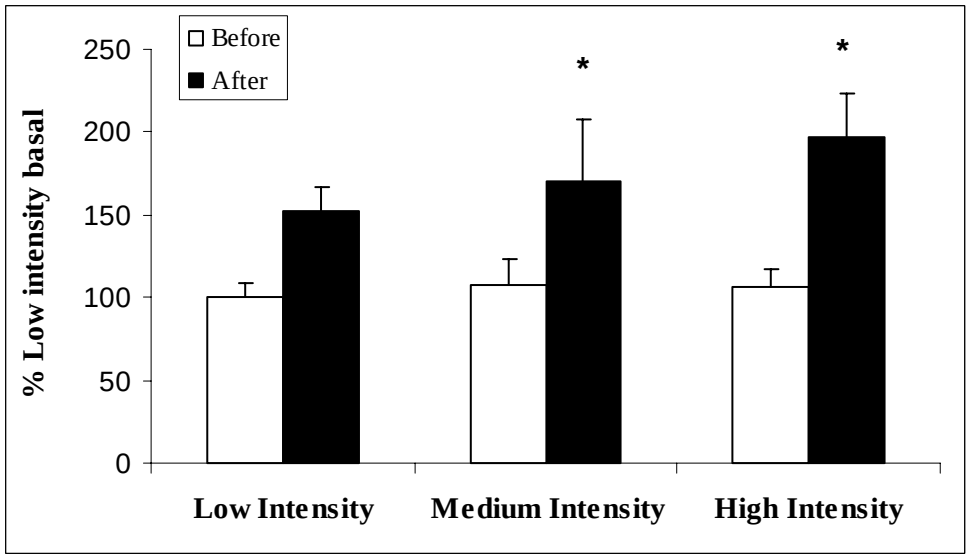
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Manuscript VIII

Supplementation with an antioxidant cocktail containing coenzyme Q prevents plasma oxidative damage induced by soccer.

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ORIGINAL ARTICLE

Supplementation with an antioxidant cocktail containing coenzyme Q prevents plasma oxidative damage induced by soccer

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Abstract The aim of the study was to determine the effects of an antioxidant supplementation, which includes coenzyme Q_{10}, on plasma and neutrophil oxidative stress and the antioxidant response after a soccer match. Nineteen voluntary male pre-professional footballers were randomly and double-blinded treated with either a multivitamin and mineral supplement ($n = 8$) or a placebo ($n = 11$). After the 3 months of supplementation, the sportsmen played a friendly soccer match of 60 min. The 3-month supplementation induced higher plasma ascorbate and coenzyme Q levels when compared to the placebo group. Antioxidant supplementation influenced plasma oxidative stress markers because they were lower in the supplemented group than in the placebo one after the match. The football match induced decreased neutrophil vitamin E levels and catalase and glutathione peroxidase activities but increased glutathione reductase activity. Antioxidant diet supplementation prevented plasma oxidative damage but did not influence the neutrophil response to a football match.

Keywords Soccer · Oxidative stress · Antioxidant supplementation · Coenzyme Q · Plasma · Neutrophils

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Introduction

Coenzyme Q_{10} is an endogenous enzyme cofactor that is produced in all living cells in humans. It functions as a catalyst in proton/electron translocation in mitochondria and lysosomes, protects mitochondria from free radical damage (Lass and Sohal 2000) and is thought to be capable of preventing programmed cell death or apoptosis (Kagan et al. 1999). Furthermore, coenzyme Q_{10} has a primarily function as antioxidant and is carried mainly by lipoproteins in the circulation (Alleva et al. 1997). Recent evidence has indicated that coenzyme Q_{10} may recycle α-tocopherol (Lass and Sohal 2000) and ascorbate (Crane 2001), may prevent prooxidant effects of α-tocopherol (Thomas et al. 1996), and may provide lipoproteins with increased resistance to oxidation. Cell signalling and gene expression have also been described as potential functions of coenzyme Q_{10} (Crane 2001).

Dietary coenzyme Q_{10} supplements contain also vitamins as vitamin E, ascorbate, and riboflavin, and some oil to facilitate the bioavailability of liposoluble compounds. Potential benefits of coenzyme Q_{10} supplementation have been recognised with particular reference to cardiovascular and neurodegenerative diseases (Langsjoen and Langsjoen 1999; Overvad et al. 1999).

Exhaustive exercise induces oxidative stress and it may impair immune response (Nieman 1994). Exercise-related immunological acute changes include release of cytokines (Petersen et al. 2001), activation of immunocompetent cell lines (Suzuki et al. 1999), neutrophil priming for acute phase response (Cannon and Blumberg 2000; Suzuki et al. 1999) and lower antioxidant enzyme levels in neutrophils (Tauler et al. 2002a). The effects of nutritional antioxidants on the endogenous antioxidant response to oxidative stress as well as on exercised-induced acute changes in

immune cell function have been pointed out (Krause et al. 2001; Morante et al. 2005; Sastre et al. 1992; Tauler et al. 2003a, b). Recent data indicate that contraction-induced ROS modulates at least some of the adaptative responses that occur in skeletal muscle following contractile activity (McArdle et al. 2005). This process involves activation of redox regulated transcription factors, such as NF-κB, leading to an increase of the expression of enzymes such as iNOS, NOS and Mn-SOD in response to intense exercise (Cases et al. 2006; Gomez-Cabrera et al. 2005; McArdle et al. 2005). High-dose antioxidant supplementation could prevent this antioxidant endogenous response to oxidative stress (Gomez-Cabrera et al. 2005). However, it is important to maintain an adequate vitamin E consumption not only to prevent liver oxidative damage but also because vitamin E plays an essential role modulating signal transduction (Morante et al. 2005). Furthermore, we have previously evidenced enhanced basal antioxidant enzyme activities in neutrophils after 3 months of antioxidant supplementation (Tauler et al. 2002b).

Because of the interest in using antioxidant nutrients as a preventive and therapeutic tool in clinical medicine and in physical activity, the aim of this study was to determine the effects of an antioxidant cocktail supplementation, which includes coenzyme Q_{10}, on plasma and neutrophil oxidative stress markers and on the antioxidant response after a football match. The influence of the 3-month supplementation on the basal levels of plasma and neutrophil antioxidant defences as well as on the basal oxidative stress markers was also analysed. In order to ensure an adequate intake of other vitamins and minerals we used capsules containing a multivitamin and mineral supplement. The use of capsules allows us to provide together adequate amounts of several vitamins and minerals, which could be very difficult by means of a simple diet manipulation.

Materials and methods

Subjects and protocol

Nineteen voluntary male pre-professional footballers participated in this study. All the subjects were informed of the purpose and demands of the study before giving their written consent to participate. The protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of CAR-Sant Cugat (Barcelona). The mean weight of the sportsmen was 75.2 ± 1.3 kg, height 178 ± 5 cm and VO_2 max 56.6 ± 3.4 mL kg^{-1} min^{-1}.

The 19 football players were randomly and double-blinded treated with either a multivitamin and mineral supplement or placebo. Compositions of the supplement are shown in Table 1. The dose of antioxidants used was the same as their RDA (when established). The eight players of the supplemented group took a capsule containing coenzyme Q and a tablet containing minerals and vitamins (Pharma Nord, Denmark) for 90 days. The 11 players of the placebo group took the same capsules and tablets as the supplemented group, but containing the placebo (Pharma Nord, Denmark). In order to ensure the compliance tablets and capsules were administered directly by one of the researchers on a daily basis. The supplement ensured the recommended allowance intake of vitamins C, E, D_3, folate, biotin, pantotenic acid, niacin, cobalamin, piridoxin and the minerals Se, Zn, Cu, Mg and Mn.

Determinations of basal haematological parameters, antioxidant enzyme activities, antioxidant vitamin levels and oxidative stress markers were made before and after the 3 months of supplementation. After the 3 months of supplementation, the sportsmen played a friendly football match (60 min), and samples were taken to determine the same parameters before and after the match. The goal-keeper did not participate in the study.

The soccer players were monitored using a pulsometer during the match. As the cardiac heart rate increases linearly to the oxygen consumption (Karvonen and Vuorimaa 1988) we can indirectly evaluate the work done during a maximal and intervallic exercise through the heart rate (Balsom et al. 1992). The relationship between the power output, the heart rate and the oxygen uptake is linear not only for the maximal values but also for the percentual, and

Table 1 Composition of the antioxidant supplement

Ingredient	Dosage	RDA (%)
β-carotene	6 mg	125
Vitamin C	60 mg	100
Vitamin E	10 mg	100
Niacin	18 mg	100
Pantothenic acid	4.0 mg	67
Vitamin B_{12}	3.0 µg	300
Vitamin B_6	2.2 mg	110
Vitamin B_2	1.6 mg	100
Vitamin B_1	1.1 mg	79
Vitamin D	2.5 µg	50
Folic acid	180 µg	90
Biotin	30 µg	20
Magnesium	100 mg	33
Zinc	7.5 mg	50
Selenium	50 µg	71
Copper	1000 µg	91
Manganese	2.5 mg	Not established
Coenzyme Q_{10}	100 mg	Not established

both can be used to monitor the training sessions (Arts and Kuipers 1994). We categorised the subjects under the perspective of the work performed during the training sessions and the competition in relation to the reference values of a progressive and maximal exercise test. Five metabolic zones are usually considered. From zone one (Z1) to zone five (Z5), the relationships to the maximal oxygen consumption were, respectively <70, 70–80, 80–90, 90–100 and 100% or higher. The pulsometer allowed to obtain the heart rate each time, which enabled us to calculate the time in which each individual worked at each intensity (zone).

Blood sampling

Blood samples were obtained from the antecubital vein of sportsmen after overnight fasting in suitable vacutainers with EDTA as anticoagulant. Neutrophils were purified following an adaptation of the method described by Boyum (1964). Blood was centrifuged at 900×*g*, at 4°C for 30 min after carefully introducing on Ficoll in a proportion of 1.5:1. The precipitate containing erythrocytes and neutrophils was incubated with ammonium chloride 0.15 M at 4°C to haemolyse erythrocytes. The suspension was centrifuged at 750×*g*, 4°C for 15 min and the supernatant was then discarded. The neutrophil phase at the bottom was washed first with ammonium chloride 0.15 M and then with PBS. Finally, neutrophils were lysed with distilled water. Neutrophil number was quantified in an automatic flow cytometer analyser Techicon H2 (Bayer) VCS system.

Plasma was obtained after centrifugation (30 min, 1,000*g*, 4°C) of another blood sample obtained as above.

Enzymatic determinations

Catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase activities were determined in neutrophils using a Shimadzu UV-2100 spectrophotometer at 37°C.

Catalase activity was measured by the spectrophotometric method of Aebi (1984) based on the decomposition of H_2O_2. Glutathione reductase activity was measured by a modification of the Goldberg and Spooner (1985) spectrophotometric method. Glutathione peroxidase (GPx) activity was measured by an adaptation of the spectrophotometric method of Flohé and Gunzler using H_2O_2 as the substrate (Flohe and Gunzler 1984). SOD activity was measured by an adaptation of the method of McCord and Fridovich (1969).

Plasma vitamins, carotenes and coenzyme Q determination

Vitamin E was determined in plasma and neutrophils. Carotenes were determined in plasma. The deep-frozen plasma or neutrophil suspensions were thawed and mixed to disperse possible precipitates. The extraction of liposoluble vitamins and carotenoids was carried out using n-hexane after deproteinisation with ethanol containing 0.2% BHT. Liposoluble vitamins and carotenoids were determined by HPLC in the n-hexane extract of plasma after drying under a nitrogen current and redissolving in ethanol. The mobile phase consisted of 550:370:80 acetonitrile:tetrahydrofuran:H_2O. The HPLC was a Shimadzu with a diode array detector and the column was a Nova Pak, C_{18}, 3.9 × 150 mm. α-tocopherol was determined at 290 nm. β-carotene and lycopene were determined at 460 and 470 nm, respectively.

Coenzyme Q was determined in another plasma extract obtained as above following an adaptation of a HPLC method previously described (Podda et al. 1999). A gradient is used consisting of a mixture of 31.7 mM ammonium formate in 80:20 methanol:H_2O and 31.7 mM ammonium formate in ethanol. The HPLC system was a Shimadzu with a diode array detector and a Nova Pak, C_{18}, 3.9 × 300 mm column. Due to the sample treatment and storage conditions all the coenzyme Q was determined as ubiquinone at 275 nm.

Plasma and neutrophil ascorbate were determined by an HPLC method with electrochemical detection (Tsao and Salimi 1982) after deproteinisation with ortho-phosphoric acid. The mobile phase consisted of 0.05 M sodium phosphate, 0.05 M sodium acetate, 189 µM dodecyltrimethylammonium chloride and 36.6 µM tetraoctylammonium bromide in 25:75 methanol: H_2O, pH 4.8. The HPLC system was a Shimadzu with a Waters Inc. electrochemical detector and a Nova Pak, C_{18}, 3.9 × 300 mm column. The potential of the chromatographic detector was set at 0.7 V versus an Ag/AgCl reference electrode.

MDA determination

The MDA as a marker of lipid peroxidation was analysed in plasma and neutrophils by a colorimetric assay kit (Calbiochem, San Diego, CA, USA). The method used is specific for MDA determination.

Carbonyl derivatives determination

Protein carbonyl derivatives were measured in plasma by an adaptation of the method of Levine et al. (1994). After deproteinising the samples with trichloroacetic acid, precipitates were resuspended with 2,4-dinitrophenylhydrazine (DNPH) 10 mM, and incubated for 60 min at 37°C. Then, samples were precipitated with 20% trichloroacetic acid and centrifuged for 10 min at 1,000*g* and 4°C. The precipitate was washed twice with ethanol:ethyl acetate (1:1) to remove free DNPH. Guanidine 6 M in phosphate buffer

2 mM, pH 2.3 was added to the precipitate, and samples were incubated for 40 min at 37°C. Finally, samples were centrifuged for 5 min at 3,000*g* at 4°C to clarify the supernatant and the absorbance was measured at 360 nm.

Statistical analysis

Statistical analysis was carried out by using a statistical package for social sciences (SPSS 10 for Windows). Results are expressed as means ± s.e.m. and $p < 0.05$ was considered statistically significant. All the data were tested for homogeneity of variance. Student's *t* test for unpaired data was used to identify differences in the physical activity performed during the soccer match (Table 2). The effects of the antioxidant supplementation on the changes induced by the football match were tested by a two-way ANOVA with antioxidant diet supplementation (S) and the football match (M) as ANOVA factors. The effects of the antioxidant supplementation and the training season on the basal parameters were also tested using a two-way ANOVA with antioxidant diet supplementation (S) and the 3 months of training (T) as factors. The sets of data in which there were significant effects were tested by the ANOVA one-way test.

Results

The physical activity performed by the football players during the match was determined (Table 2). No differences were observed during the match between the placebo and the supplemented group in the energy consumed, the mean cardiac frequency and the time expended in each exercise intensity zone (from Z1 to Z5). Furthermore, no difference was found in the time expended at 80–100% VO_2 max (the anaerobic metabolism zones, Z3–Z5) which was about 70% in both groups. These observations allowed us to compare the results obtained in the placebo and in the supplemented groups.

Table 2 Physical activity performed during the match

	Placebo	Supplemented
Energy (kcal)	924 ± 34	935 ± 57
Fc med (beats/min)	163 ± 4	165 ± 5
Z1 (%)	8.1 ± 3.2	7.6 ± 3.8
Z2 (%)	21.9 ± 5.5	24.6 ± 7.8
Z3 (%)	40.7 ± 5.7	39.4 ± 7.5
Z4 (%)	27.5 ± 8.7	26.0 ± 10.7
Z5 (%)	1.8 ± 1.0	2.4 ± 1.2

No significant differences were found between groups (Student *t* test for unpaired data, $p < 0.05$). *Z* values are expressed as percentage of time expended at each metabolic zone. Metabolic zones are defined in relation to maximal oxygen uptake: Z1 < 70%, Z2: 70–80%, Z3: 80–90%, Z4: 90–100%, Z5: 100% or higher

Table 3 shows the effect of diet supplementation on the plasma and neutrophil basal levels of antioxidant metabolites. The 3-month supplementation induced higher basal plasmatic levels of coenzyme Q (29%) in the supplemented group than in the placebo one. No significant changes were observed in the basal plasmatic levels of vitamin E, carotene and lycopene.

The ANOVA analysis of the plasmatic antioxidant levels before and after the match (Table 4) revealed a significant effect of the supplementation on the ascorbate and coenzyme Q levels. As a consequence of the supplementation, the basal coenzyme Q levels in the supplemented group were higher than in the placebo one. On the other hand, the non-significant increases observed after the game in the ascorbate levels induced higher final levels in the supplemented group than in the placebo one.

Table 3 Effects of antioxidant supplementation of the diet on basal plasma and neutrophil antioxidants

	Initial		Final		ANOVA		
	Placebo	Supplemented	Placebo	Supplemented	*S* × *T*	*S*	*T*
Coenzyme Q (μg/L)	297 ± 24	306 ± 25	278 ± 18	359 ± 15[a]		*	
α-Tocopherol (μM)	21.5 ± 1.2	20.4 ± 1.2	23.2 ± 1.2	21.8 ± 1.5			
Carotene (μg/L)	340 ± 80	222 ± 48	477 ± 162	519 ± 171			
Lycopene (μg/L)	453 ± 74	335 ± 35	530 ± 68	422 ± 41			
Neutrophils							
Ascorbate (mM)	1.16 ± 0.13	0.99 ± 0.06	0.84 ± 0.11[b]	0.83 ± 0.12[b]			*
α-Tocopherol (μM)	93.2 ± 5.4	101 ± 7	104 ± 14	98.8 ± 6.4			

Two way ANOVA

S supplementation, *T* time (3 months supplementation), *S* × *T* supplementation time interaction

[a] Indicates significant differences placebo versus supplemented

[b] Indicates significant differences initial versus final

Table 4 Effects of a football match and of the antioxidant supplementation on plasma and neutrophil antioxidant levels

	Before		After		ANOVA		
	Placebo	Supplemented	Placebo	Supplemented	$S \times M$	S	M
Plasma							
Ascorbate (μM)	30.9 ± 3.4	40.6 ± 5.08	37.1 ± 4.2	52.4 ± 7.2[a]		*	
Coenzyme Q (μg/L)	278 ± 18	359 ± 15[a]	278 ± 30	318 ± 23		*	
α-Tocopherol (μM)	23.2 ± 1.2	21.8 ± 1.5	23.4 ± 1.4	22.6 ± 1.6			
Carotene (μg/L)	477 ± 162	519 ± 171	427 ± 156	443 ± 171			
Lycopene (μg/L)	530 ± 68	422 ± 41	504 ± 55	430 ± 47			
Neutrophils							
Ascorbate (mM)	0.84 ± 0.11	0.83 ± 0.12	0.82 ± 0.05	0.77 ± 0.07			
α-Tocopherol (μM)	104 ± 14	98.8 ± 6.4	76.7 ± 4.7[b]	76.3 ± 8.6			*

Two way ANOVA

S supplementation, M match, $S \times M$ supplementation match interaction

[a] Indicates significant differences placebo versus supplemented

[b] Indicates significant differences before versus after

A significant effect of the time factor was observed in the basal neutrophil ascorbate levels (Table 3). Significant decreases were observed after the 3 months of study both in the placebo (28%) and in the supplemented (16%) groups. The basal neutrophil vitamin E concentrations did not change throughout the study. However, the match induced significant decreases in neutrophil vitamin E in the placebo and in the supplemented groups (Table 3).

Basal neutrophil counts are maintained during the 3 months of training and competition (Table 5). When changes in the basal neutrophil antioxidant enzyme activities were analysed, a significant effect of the time factor was observed on the glutathione reductase activity, decreasing this activity in both groups at the end of the study. Basal catalase and glutathione peroxidase activities did not change along the study.

The football match performed after the supplementation induced higher neutrophil counts (Table 6). The circulating number of neutrophils increased about 67% in the placebo group and about 85% in the supplemented one. The football match influenced the neutrophil activities of catalase, glutathione peroxidase and glutathione reductase. Catalase and glutathione peroxidase activities decreased significantly in both placebo and supplemented groups. An increase in the neutrophil glutathione reductase activity was observed after the football game. This increase was significant only in the placebo group. SOD activity did not change along the study.

Figure 1 shows the basal MDA levels in plasma and neutrophils before and after the supplementation period. Basal plasma MDA levels increased both in the placebo (165%) and in the supplemented (189%) groups at the end of the study. The supplementation did not influence these basal plasma MDA levels because they were similar in both groups after the 3 months of study. When the changes in plasma MDA levels during the football match were analysed (Fig. 2) a significant effect of the supplementation was found: the values after the match were higher (100%) in the placebo than in the supplemented group. Neutrophil MDA levels did not change throughout the study.

Table 5 Effects of the supplementation on the basal neutrophil number and neutrophil basal antioxidant enzyme activities

	Initial		Final		ANOVA		
	Placebo	Supplemented	Placebo	Supplemented	$S \times T$	S	T
10^3 cells/μL blood	3.54 ± 0.44	3.30 ± 0.29	3.20 ± 0.41	3.45 ± 0.31			
Catalase (K/10^9 cells)	15.2 ± 1.8	16.4 ± 2.0	17.8 ± 1.4	16.6 ± 1.5			
GPx (nkat/10^9 cells)	28.3 ± 2.8	25.9 ± 2.3	27.4 ± 2.3	23.4 ± 2.3			
G Red (nkat/10^9 cells)	91.9 ± 33.1	118 ± 29	46.7 ± 6.1	42.7 ± 7.3[a]			*

Two way ANOVA

S supplementation, T time (3 months supplementation), $S \times T$ supplementation time interaction

[a] Indicates significant differences initial versus final

Table 6 Effects of the football match on the neutrophil number and neutrophil antioxidant enzyme activities after the antioxidant supplementation

	Before		After		ANOVA		
	Placebo	Supplemented	Placebo	Supplemented	$S \times M$	S	M
10^3 cells/μL blood	3.20 ± 0.41	3.45 ± 0.31	5.34 ± 0.49[a]	6.38 ± 1.31[a]			*
Catalase (K/10^9 cells)	17.8 ± 1.4	16.6 ± 1.5	10.8 ± 1.2[a]	11.5 ± 1.2[a]			*
GPx (nkat/10^9 cells)	27.4 ± 2.3	23.4 ± 2.3	17.4 ± 1.6[a]	16.4 ± 2.3[a]			*
G Red (nkat/10^9 cells)	46.7 ± 6.1	42.7 ± 7.3	144 ± 35[a]	100 ± 34			*
SOD (pkat/10^9 cells)	86.3 ± 14.5	78.4 ± 13.5	83.1 ± 8.9	85.4 ± 25.9			

Two way ANOVA

S supplementation, *M* match, $S \times M$ supplementation match interaction

[a] Indicates significant differences before versus after

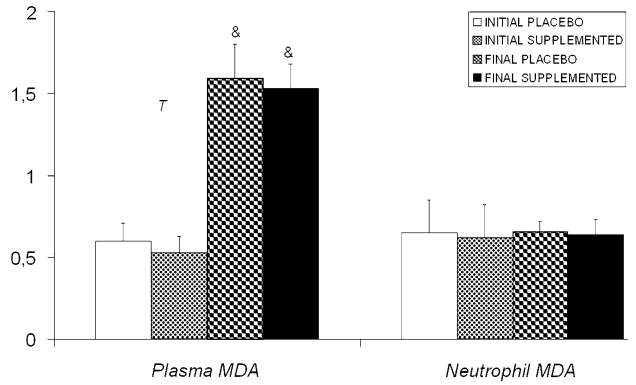

Fig. 1 Effects of the antioxidant supplementation on basal plasma and neutrophil MDA levels. Two way ANOVA. *S* supplementation; *T* time (3 months supplementation). $S \times T$ supplementation time interaction. *Hash symbol* indicates significant differences placebo versus supplemented; *ambersand* indicates significant differences initial versus final

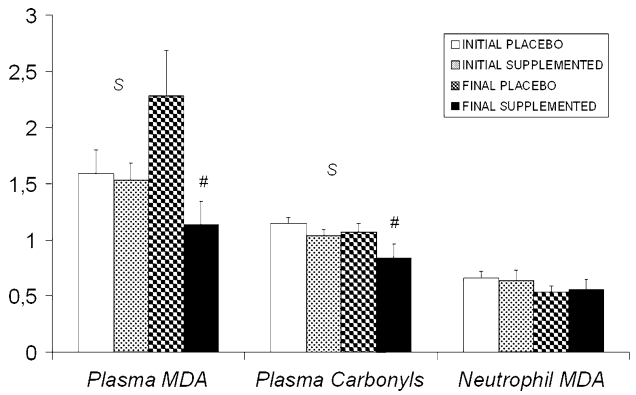

Fig. 2 Effects of a football match and of the antioxidant supplementation of the diet on the oxidative damage markers in plasma and neutrophils. Two way ANOVA. *S* supplementation; *T* time (3 months supplementation). $S \times T$ supplementation time interaction. *Hash symbol* indicates significant differences placebo versus supplemented; *ambersand* indicates significant differences initial versus final

Plasma protein carbonyl derivatives, as oxidative stress marker, were also measured before and after the football match (Fig. 2). The supplementation did not induce any differences in the pre-match protein carbonyl concentrations. However, concentrations were significantly lower in the supplemented group than in the placebo one after the match.

Discussion

Plasma antioxidant vitamins and other nutrient levels of all sportsmen participating in the study were within the range of well-nourished people (Tauler et al. 2002b). Diet supplementation with the antioxidant cocktail induced a real increase in the plasma levels of coenzyme Q. Under normal conditions plasma coenzyme Q concentrations are not significantly affected by dietary components such as dairy products, eggs, fish and vegetables. The nutritional habits of the football players participating in the study did not influence the basal levels of antioxidant nutrients in plasma. Coenzyme Q supplementation leads to increases in plasma coenzyme Q concentrations, the extent of which depends upon the dosage, duration and also the type of formulation (Bhagavan and Chopra 2006). The controlled trials seems to report a dose-dependent increase in plasma coenzyme Q in function of the chronic daily dose of coenzyme Q administered up to the daily dose of 200 mg (Bhagavan and Chopra 2006). Sportsmen in the present study took a 100 mg/day dose of coenzyme Q, but plasma concentration increased only about 25%; this increase is lower than those described by others (Kaikkonen et al. 2002; Niklowitz et al. 2004). However, and in accordance with the lower increases in other antioxidant nutrients as vitamin E and vitamin C observed after their supplementation (Tauler et al. 2002b), sportsmen present higher resistance to increase plasma coenzyme Q_{10} concentration than general population. It has been postulated that the reduced form of coenzyme Q_{10}, together with α-tocopherol, prevents lipid peroxidation in plasma lipoproteins and biological membranes (Ernster and Forsmark-Andree 1993). The lower increase in coenzyme Q_{10} observed in plasma of sportsmen

could indicate higher coenzyme Q_{10} cellular utilization by sportsmen than by general population.

The training and competition sessions resulted in increased basal oxidative stress as indicated by the increased MDA plasma levels after the 3 months of study. The antioxidant supplementation did not prevent this basal increased oxidative stress because similar increases were observed in both groups. It has been previously reported that an antioxidant supplementation with vitamin C, E and β-carotene decreased the lipoperoxide levels in basketball players (Schroder et al. 2000). Differences in the supplementation, in the oxidative stress markers analysed and in the competition and training sessions developed by the sportsmen could explain the differences in the results obtained. The molecular damage produced by ROS is parallel to the activation of the endogenous antioxidant defences (Gomez-Cabrera et al. 2005; Jackson 1999; Sureda et al. 2005). In a similar way, free radicals could be involved in the muscle adaptations to exercise in skeletal muscle; some ROS production is needed to attain optimal muscular isometric force production (Reid 2001). The basal plasma molecular damage increased during the study; this increase could be related with the muscle adaptations to exercise mediated by ROS. The surplus intake of antioxidants with the supplement did not influence the adaptations to exercise.

DT-diaphorase (NAD(P)H: quinone acceptor oxidoreductase) is an inducible antioxidant enzyme that maintains the reduced antioxidant form of coenzyme Q_{10} in membrane systems and to protect against xenobiotics which could generate ROS (Radak et al. 2000). DT-diaphorase activity increased in response to regular exercise in rats (Radak et al. 1999, 2000) and in response to chronic administration of hydrogen peroxide (Radak et al. 2000). In the present study DT-diaphorase activity has not been determined. Recent studies reported that antioxidant supplementation could prevent endogenous antioxidant adaptations to increased ROS production (Gomez-Cabrera et al. 2005). However, it has been also indicated that molecular damage produced by ROS is parallel to the activation of the endogenous antioxidant defences (Gomez-Cabrera et al. 2005; Jackson 1999; Sureda et al. 2005). Because a similar increase in basal plasma MDA levels had been found in both groups after the 3 months of supplementation in the present study, we could suppose that DT-diaphorase activity could be increased in both groups in parallel to increased MDA levels as a result of regular exercise as it has been indicated previously (Radak et al. 1999, 2000). However, additional studies are necessary in order to determine the effects of a supplementation with coenzyme Q on DT-diaphorase activity.

Increases in plasma MDA levels after exercise are widely shown in the literature (Miyazaki et al. 2001; Tauler et al. 2006). We appreciate a high variability in the post-exercise MDA values between sportsmen, probably because the intensity of the exercise developed by the football players depends on their position. The maintenance of protein carbonyl derivatives after the football match is in agreement with the findings of others (Miyazaki et al. 2001). A strong correlation between serum and urinary protein carbonyl derivatives was found in a previous study (Radak et al. 2003), suggesting that the filtration of carbonylated proteins could prevent its accumulation in plasma. Furthermore, results obtained in the present study revealed an influence of antioxidant levels because carbonyl derivatives were lower in the supplemented group than in the placebo one after the match. In fact, antioxidant supplementation influenced not only carbonyl derivative but also MDA levels in plasma. Taken together, we can suggest that even with low values of oxidative stress markers, the moderate antioxidant supplementation induced beneficial effects as indicated by the lower post-exercise MDA and carbonyl plasma levels in the supplemented group.

The football match decreased the neutrophil α-tocopherol but maintained the plasma concentrations in both groups. Changes in neutrophil tocopherol levels could be related with its availability but also with the exercise-induced oxidative stress. Previous studies have shown increases in neutrophil tocopherol after an acute bout of exercise, especially in supplemented sportsmen, but related to increases in plasma tocopherol (Cases et al. 2005). Increases in plasma tocopherol are, in turn, related with the VLDL output from the liver and with the triglyceride mobilisation when the intensity and duration of the exercise activate these processes (Aguilo et al. 2005). Higher oxidative stress levels or longer exercises could activate mechanisms leading to increased neutrophil vitamin E levels. As far as we know mechanisms underlying the neutrophil vitamin E uptake are not well known. Decreased vitamin E levels could be related to the maintenance of MDA levels in neutrophils. Vitamin E could play an essential role in neutrophils preventing higher oxidative damage as it is indicated by the lack of increases in MDA levels. It seems that neutrophil antioxidant defences are effective preventing exercise-induced oxidative damage because no changes in oxidative stress markers have been reported after several exercises (Sureda et al. 2005).

Interactions between neutrophil antioxidant enzyme activities and training but also with antioxidant supplementations have been previously reported (Tauler et al. 2002b). A significant decrease was observed in the basal neutrophil glutathione reductase activity during the study. We previously reported a significant decrease in neutrophil glutathione reductase activity after 3 months of training and competitions (Tauler et al. 2002b). However, this activity decreased only in the placebo group whereas it was

maintained in the antioxidant supplemented one (Tauler et al. 2002b). In the present study the moderate antioxidant supplementation did not prevent the decrease in glutathione reductase activity. Decreases in glutathione reductase activities have been related to increased requirements of riboflavin in trained sportsmen (Ohno et al. 1988), because this enzyme is highly dependent on this vitamin. This is rather unlikely in this study because we ensured that the demands of riboflavin were covered with the supplementation. Thus, other causes such as oxidative modifications in the enzymatic protein should be considered as the main factor inducing the activity decrease (Tauler et al. 2002b).

Exercise induces an acute phase immune response (APIR) similar to the one induced by an infection (Cannon and Blumberg 2000). Increased neutrophil circulating counts as well as decreased neutrophil antioxidant enzyme activities (Tauler et al. 2002a) have been reported during this APIR. The football match induced an APIR but not as important as the ones observed after more intense exercises (Tauler et al. 2002a) as it is indicated by the slight changes observed in parameters such as the neutrophil number. The antioxidant diet supplementation influenced the APIR induced by exercise, producing higher decreases of antioxidant enzyme activities in neutrophils (Tauler et al. 2003b). However, the moderate antioxidant diet supplementation in this study did not influence neutrophil antioxidant response to the exercise because similar pictures were observed after the match in both the placebo and the supplemented groups. Catalase and glutathione peroxidase, the hydrogen peroxide removing enzymes, decreased their activities after the football match. However, glutathione reductase increased its activity, indicating enhanced glutathione regeneration in neutrophils after the football match. This increased glutathione reductase activity after the match could evidence a good availability of riboflavin; then, the basal decrease in this enzyme activity after the period of training and competition must be attributed to decreased enzyme levels. The increased glutathione reductase and the decreased glutathione peroxidase activities after the match could indicate that this enhanced glutathione regeneration is not induced by a higher rate in its consumption to remove H_2O_2, suggesting another role for glutathione in the neutrophil antioxidant defence during exercise. This role could be related to the recycling of ascorbate from its oxidised form, dehydroascorbate. Neutrophils preferentially uptake dehydroascorbate and then it is reduced to ascorbate by both glutathione-dependent and glutathione-independent systems (Welch et al. 1995). Then, the maintenance of neutrophil ascorbate concentration after the football match is in accordance with this potential role for glutathione and with the increased glutathione reductase activity.

In summary, the moderate antioxidant supplementation of the diet for 3 months using a multivitamin and mineral cocktail prevented the plasma oxidative molecular damage induced by a football match without influencing the antioxidant adaptations induced by exercise. The supplementation did not influence the antioxidant response or the oxidative stress makers in neutrophils.

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Manuscript IX

A soccer match enhances lymphocyte capability to produce ROS and induces oxidative damage. Effects of low doses of antioxidant supplementation of the diet.

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A soccer match enhances lymphocyte capability to produce ROS and induces oxidative damage. Effects of low doses of antioxidant supplementation of the diet.

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Abstract
Soccer-associated oxidative stress has been barely studied. The aims of this study were to establish i) the effect of a soccer training match and ii) the effect of a diet supplementation with a multivitamin complex and coenzyme Q during three months of soccer training on the prooxidant and antioxidant status of lymphocytes. In a randomized, double-blind trial, nineteen male pre-professional soccer players were treated with either an antioxidant nutrient cocktail or placebo for 90 days. After this period the sportsmen played a soccer match lasting 60 minutes. All determinations were made in basal conditions before and after the training period and after the match. Basal lymphocyte H_2O_2 production did not change after the three months of training. Catalase activity decreased (about 50%) after the three months, while glutathione reductase increased its activity (150-200%) both in placebo and supplemented groups. Basal ascorbate levels were maintained during the training period while α-tocopherol and MDA decreased (about 40%) in both groups. The match increased H_2O_2 production (180%) in both groups when the lymphocytes were stimulated with PMA and it also increased MDA levels (150%). Antioxidant enzyme activities and antioxidant vitamin levels were maintained before and after the match. Regular soccer training modifies the lymphocyte strategy to eliminate ROS and increases protection against oxidative damage. A friendly soccer match raises lymphocyte capacity to produce ROS and oxidative damage, but it is not enough to induce a defensive response, thus leading to a situation of post-exercise oxidative stress. Supplementation with low dosages of antioxidant vitamins and coenzyme Q does not modify the endogenous antioxidant response to training.

Keywords: Oxidative stress, exercise, coenzyme Q, ascorbate, tocopherol.

Introduction

Physical exercise increases oxygen consumption and reactive oxygen species (ROS) formation (Ji, 1995). Although the body contains an elaborate antioxidant defense system, when ROS production is excessive an adequate defense may be overwhelmed by the ROS. This condition leads to a situation of oxidative stress, in which the free radicals can attack polyunsaturated fatty acids, proteins and nucleic acids leading to cell and tissue damage (Banerjee et al., 2003). Lymphocytes have been shown to be a good model to study oxidative stress. Antioxidant enzyme activities increase in response to oxidative stress in lymphocytes. After a cycling stage lymphocytes have been shown to raise inducible nitric oxide synthase (iNOS) levels and superoxide dismutase (SOD) activity (Sureda et al., 2005).

Several studies have examined the effects of antioxidant supplementations (mainly vitamin C, vitamin E and beta-carotene) on the antioxidant status of sportsmen (Clarkson and Thompson, 2000; Ji, 1995). The intake of vitamins in professional and amateur sportsmen is in the range of the recommended dietary allowances (RDA), although vitamin C intake is usually increased above the RDA, ranging from 110 to 200 mg/day (Sureda et al., 2004). Supplementation with 1 g/day of vitamin C induced NO synthesis in erythrocytes after an apnea diving session (Sureda et al., 2004), but at the same time attenuated NO increase and SOD activation in lymphocytes after apnea diving (Sureda et al., 2004) and after a duathlon competition (Tauler et al., 2003). Supplementation with 50 mg/day of vitamin E in athletes who already comply with the RDA for vitamin E has been shown to improve the vitamin distribution in

lymphocytes and neutrophils after a half marathon race (Cases et al., 2005). Sportsmen supplemented with high doses of antioxidant vitamins showed improved response of antioxidant enzymes in lymphocytes (Tauler et al., 2005) and enhanced oxidative burst in neutrophils (Robson et al., 2003) after acute exercise. Coenzyme Q_{10} is an endogenous enzyme cofactor that is produced in all living cells in humans. It functions as a catalyst in proton/electron translocation in mitochondria and lysosomes (Nohl et al., 2003) and protects mitochondria from free radical damage (Lass and Sohal, 2000). Carried mainly by lipoproteins in the circulation, coenzyme Q_{10} can also act as an antioxidant (Alleva et al., 1997). Recent evidence has indicated that coenzyme Q_{10} may recycle α-tocopherol (Lass and Sohal, 2000) and ascorbate (Crane, 2001), may prevent prooxidant effects of α-tocopherol (Thomas et al., 1996), and may provide lipoproteins with increased resistance to oxidation. Potential benefits of coenzyme Q_{10} supplementation have been recognized with particular reference to cardiovascular and neurodegenerative diseases (Overvad et al., 1999). Previous studies concerning antioxidant vitamin supplementations were performed with high concentrations of the antioxidant vitamins in the supplemented groups, but the effects of supplementation with doses of vitamins closer to their RDA and with coenzyme Q are not yet known.

Soccer is one of the most popular sports worldwide, but few studies have focused on the effect of soccer training on the antioxidant status of players (Brites et al., 1999; Metin et al., 2003). To date, there are no data concerning the effects of an acute bout of soccer practice on the antioxidant status of players. The aims of this study were to establish: i) the effect of three months of soccer training, ii) the effect of a soccer training match, and iii) the effect of supplementation with low doses of vitamins and coenzyme Q on the lymphocyte oxidant and antioxidant status of sportsmen who already exceeded the RDA for vitamins C and E.

Materials and methods

Subjects and protocol

Nineteen voluntary male pre-professional soccer players belonging to a Spanish soccer team participated in this study. All the subjects were informed of the purpose and demands of the study before giving their written consent to participate. The protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of CAR-Sant Cugat (Barcelona).

The nineteen players were randomly and double-blind treated with either multivitamin and mineral cocktail (composition shown in Table 1) or placebo. The supplemented group initially consisted of 9 subjects, but one of them left the study and was not considered in any analysis. The eight players of the supplemented group took the supplementation 6 days a week just before training or competition. The supplement consisted of one capsule containing coenzyme Q_{10} (BioQ10, Pharma Nord) and one tablet containing the vitamin supplementation (Bio-Antioxidant 2.1 Pharma Nord). The eleven players of the placebo group took the same capsules (containing soy bean oil as bulking agent) and tablets (containing microcrystalline cellulose as bulking agent) as the supplemented group, but without any of the nutrients present in the supplement.

The anthropometric parameters of both groups of sportsmen are shown in Table 2. Subjects were aged 19-21 and 18-21 years in the placebo and supplemented groups, respectively. Height was determined using a mobile anthropometer (KaWe 44444, Kirchner & Wilhelm GmbH Co. KG, Asperg, Germany) to the nearest mm, with the

Table 1. Total composition of the antioxidant supplement.

Ingredient	Dosage	RDA (%)[a]
Vitamin A	1000 μg	125
Vitamin C	60 mg	100
Vitamin E	10 mg	100
Niacin	18 mg	100
Pantothenic acid	4.0 mg	80
Vitamin B12	3.0 μg	125
Vitamin B6	2.2 mg	147
Vitamin B2	1.6 mg	100
Vitamin B1	1.1 mg	92
Vitamin D	2.5 μg	50
Folic acid	180 μg	45
Biotin	30 μg	100
Magnesium	100 mg	25
Zinc	7.5 mg	50
Selenium	50 μg	71
Copper	1000 μg	91[b]
Manganese	2.5 mg	Not established
Co-enzyme Q_{10}	100 mg	Not established

[a] Calculated upon Spanish IDRs (Recommended Daily Intakes) (Ortega et al., 2004)
[b] Calculated upon European PRI (Population Reference Intakes) (1993)
RDA: Recommended Dietary Allowances

subject's head in the Frankfurt plane. Body weight was determined to the nearest 100 g using a digital scale (Tefal, sc 9210, France). The subjects were weighed in bare feet and light underwear, which was accounted for by subtracting 200–400 g from the measured weight. Kinanthropometric measures were performed following the protocol of Ross and Marfell-Jones (Ross and Marfell-Jones, 1991). Body fat mass and muscle mass fractions were calculated following Drinkwater and Ross methodology (Drinkwater and Ross, 1980).

Supplementation was carried out during 3 months of training and competition. All the subjects performed 4 training sessions a week consisting of 120 minutes of soccer practice, and 2 training sessions a week of 90 minutes' physical preparation or muscular recovery, depending on the time of season. A soccer match was played once a week. VO_2max was estimated after the 20 m shuttle run test at the end of the training period (Leger and Lambert, 1982). Briefly, subjects ran back and forth on a 20 m course following a sound signal emitted from a prerecorded tape. Frequency of the sound signals was increased so that the running speed increased by 0.5 km/h each minute from a starting speed of 8.5 km/h. When the subject could no longer follow the pace, the test finished and VO_2max was estimated.

Determinations of hematological parameters and enzymatic activities were made in basal conditions before and after the three months of supplementation. After the three months of supplementation the sportsmen played a training soccer match lasting 60 minutes, and samples were taken to determine the same parameters before and 1 hour after the match.

The soccer players were monitored using a pulsometer during the match. As the cardiac heart rate increases linearly to the oxygen consumption (Karvonen and Vuorimaa, 1988) we can indirectly evaluate the work done during a maximal and intervallic exercise through the heart rate (Balsom et al., 1992). The relationship between the power output, the heart rate and the oxygen uptake is linear not only for the maximal values but also for the percentual, and both can be used to monitor the training sessions (Arts and Kuipers, 1994). The subjects were categorized under the perspective of the work performed during the match in relation to the reference values of VO_2max previously measured. The pulsometer allows us to obtain the heart rate each time and then we can extrapolate the time each individual performed at each intensity. Five metabolic zones are usually considered. From zone one (Z1) to zone five (Z5), the relation to the maximal oxygen consumption are, respectively, <70%, 70-80%, 80-90%, 90-100% and 100% or higher.

Table 2. Anthropometric parameters of the soccer players.

	Placebo	Supplemented
Age (years)	20.0 ± 0.2	19.5 ± 0.4
Weight (kg)	72.8 ± 1.7	77.6 ± 2.1
Height (cm)	175 ± 2	179 ± 2
Muscle mass (%)	44.1 ± 0.5	43.9 ± 0.7
Body fat mass (%)	9.6 ± 0.4	10.0 ± 0.5

Experimental procedure

Venous blood samples were obtained from the antecubital vein of soccer players in suitable vacutainers with EDTA as anticoagulant. Lymphocyte fraction was purified from whole blood following an adaptation of the method described by Boyum (Boyum, 1964; Ferrer et al., 2007) and quantified in an automatic flow cytometer analyzer Technicon H2 (Bayer) VCS system. Hydrogen peroxide production, antioxidant enzyme activities and ascorbate, α-tocopherol and MDA levels were measured in lymphocytes.

Hydrogen peroxide production

H_2O_2 production in lymphocytes was measured before and after stimulation with phorbol myristate acetate (PMA) using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as indicator. DCFH-DA (30 μg/ml) in PBS was added to a 96-well microplate containing 50 μl lymphocyte suspension. PMA (3 μM) prepared in HBSS or HBSS alone was added to the wells and the fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37ºC for 1 hour in a FLx800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc.).

Enzymatic determinations

All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37ºC. Catalase (CAT) activity was measured by the spectrophotometric method of Aebi based on the decomposition of H_2O_2 (Aebi, 1984). Glutathione peroxidase (GP) activity was measured following the spectrophotometric method of Flohé and Gunzler (Flohe and Gunzler, 1984). Glutathione reductase (GR) activity was measured by a modification of the Goldberg and Spooner spectrophotometric method (Goldberg and Spooner,

1985) using 1mM EDTA, 200 mM phosphate buffer, pH 7.0. Superoxide dismutase (SOD) activity was measured by an adaptation of the method of McCord and Fridovich (McCord and Fridovich, 1969) monitoring the reduction of cytochrome c using 0.02 mM cytochrome c, 0.05 mM xanthine and sufficient xanthine oxidase to produce a rate of reduction of cytochrome c of 0.080 absorbance unit per min.

Vitamin determinations

Vitamin E was determined in lymphocytes. The extraction of liposoluble vitamins was carried out using n-hexane after deproteinization with ethanol containing 0.2% BHT. Vitamin E was determined by HPLC in the n-hexane extract after drying under a nitrogen current and redissolving in ethanol. The mobile phase consisted of 550:370:80 acetonitrile:tetrahydrofuran:H_2O. The HPLC was a Shimadzu with a diode array detector and the column was a Nova Pak, C_{18}, 3.9x150 mm. α-tocopherol was determined at 290 nm.

Ascorbate was determined by an HPLC method with electrochemical detection (Tsao and Salimi, 1982) after deproteinization with meta-phosphoric acid. The mobile phase consisted of 0.05 M sodium phosphate, 0.05 M sodium acetate, 189 μM dodecyltrimethylammonium chloride and 36.6 μM tetraoctylammonium bromide in 25:75 methanol: H_2O, pH 4.8. The HPLC system was a Shimadzu with a Waters Inc electrochemical detector and a Nova Pak, C_{18}, 3.9x300 mm column. The potential of the chromatographic detector was set at 0.7 V versus an Ag/AgCl reference electrode.

MDA determination

MDA as a marker of lipid peroxidation was analyzed in lymphocytes by a colorimetric assay kit (Calbiochem, San Diego, CA, USA). The method used is specific for MDA determination.

Statistical analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 12.0 for Windows). Results are expressed as mean ± SEM and $P<0.05$ was considered statistically significant. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analyzed were time (T), supplementation (S) and match (M). When significant effects of time (T), supplementation (S) or match (M) were found, Student's t-test for unpaired data was used to determine the differences between the groups involved. When significant interactions were found, one-way ANOVA was used to determine the differences between the groups involved.

Table 3. Daily nutrient intake of the sportsmen prior to supplementation.

Nutrient	Dosage	RDA (%)[a]
Energy intake	3028 kcal	93[b]
Water	1750 ml	
Total protein	154 g	240
Vegetable protein	47.3 g	
Animal protein	107 g	
Carbohydrates	382 g	
Lipids	97.8 g	
Retinoids	343 μg	
Carotenoids	6624 μg	
Vitamin A	1671 μg	141
Vitamin E	17.3 mg	143
Vitamin D	13,6 μg	220
Vitamin C	161 mg	227
Zn	11.7 mg	66
Fe	15.6 mg	132

[a] Calculated upon Spanish IDRs (Recommended Daily Intakes) (Ortega et al., 2004)
[b] Energy expenditure calculated with the equation [(15.3 x Weight) + 679] and corrected with an intense activity coefficient (x 1.78) (1985).
RDA: Recommended Dietary Allowances

Results

Effects of training and a multivitamin and coenzyme Q_{10} supplementation on the basal status of soccer players

In the first part of this study we compared the basal status of the sportsmen before and after the three months of supplementation. All sportsmen had a controlled diet and expended similar periods of training and competition as evidenced by the existence of similar sportive and nutritional habits derived from their belonging to a soccer club such as FC Barcelona. The diet of the players was highly controlled by the club staff and all players were provided with the same food consumption guides during the season. Daily nutrient recommendations of the sportsmen during the season are shown in Table 3. The recommended intakes for vitamins A, E, D and C were already higher than the recommended dietary allowances. No additional food intake was allowed and players agreed to carry

Table 4. Effects of an antioxidant supplementation on the basal lymphocyte antioxidant enzymatic activities.

		Before supplementation	After supplementation	ANOVA		
				T	S	TxS
Lymphocytes (10^6/ml)						
	Placebo	2.76 ± 0.23	2.41 ± 0.17			
	Supplemented	2.57 ± 0.13	2.56 ± 0.32			
Catalase (K/10^9 cells)						
	Placebo	12.9 ± 1.6	5.73 + 0.55*	x		
	Supplemented	13.4 ± 2.1	6.42 ± 0.88*			
Glutathione peroxidase (nkat/10^9 cells)	**Placebo**	61.6 ± 6.8	67.3 ± 7.6			
	Supplemented	57.1 ± 6.6	81.0 ± 16.2			
Glutathione reductase (nkat/10^9 cells)	**Placebo**	31.9 ± 4.2	59.7 ± 6.6*	x		
	Supplemented	36.4 ± 3.6	50.0 ± 4.4			
Superoxide dismutase (pkat/10^9 cells)	**Placebo**	61.4 ± 8.5	69.8 ± 7.0			
	Supplemented	66.5 ± 8.7	87.2 ± 8.2			

Statistical analysis: Two-way ANOVA. (T) significant effect of time (3 months). (S) significant effect of supplementation. (TxS) significant interaction between the ANOVA factors. (*) significant differences between before and after, $p < 0.05$.

out the recommendations, so no additional food recordings were performed.

H_2O_2 production rose about 4-fold when lymphocytes were stimulated with PMA, but was unaffected by the three months of training (Figure 1). The supplementation did not affect these parameters either. Table 4 shows the basal number of circulating lymphocytes and the basal antioxidant enzyme activities before and after the three months of training and supplementation. The three months of training did not affect the basal number of circulating lymphocytes. Catalase activity significantly decreased about 50% both in the placebo and supplemented group, while glutathione reductase activity was significantly increased mainly in the placebo group. Glutathione peroxidase and SOD activities did not change significantly.

The basal levels of antioxidant vitamins are shown in Table 5. Lymphocyte ascorbate levels did not change after the three months, but α-tocopherol levels decreased about 1.5-fold in both the placebo and supplemented group. This mobilization of tocopherol might be related to the decrease in MDA levels (Table 5) observed in both groups after the three months of training.

Effects of a soccer match on the oxidant and antioxidant lymphocyte status

After the three months of supplementation a soccer match was played, and the same parameters were measured before and after. The work performed by the sportsmen during the match is presented in Table 6. No differences were observed between placebo and supplemented groups in the energy consumed (kcal/match), the mean cardiac frequency (Fcmed), or the time performing at 80-100% VO_2max in the anaerobic metabolism zones (Z3-Z5, data extrapolated from the cardiac frequencies) during the soccer match.

Lymphocyte H_2O_2 production was not different between groups, although the match significantly increased the production in both groups when lymphocytes were stimulated with PMA (Figure 2).

No significant lymphopenia was observed after the match (Table 7). Basal antioxidant enzyme activities (Table 7) and levels of antioxidant vitamins (Table 8) were maintained, although MDA levels were increased after the match. The supplementation showed no effects on any of these parameters.

Table 5. Effects of an antioxidant supplementation on the basal levels of low molecular weight antioxidants and malondialdehyde on lymphocytes.

		Before supplementation	After supplementation	ANOVA		
				T	S	TxS
Ascorbate (mM)						
	Placebo	2.88 ± 0.48	2.56 ± 0.17			
	Supplemented	3.37 ± 0.36	3.16 ± 0.43			
α-tocopherol (mM)						
	Placebo	0.47 ± 0.08	0.27 ± 0.02*	x		
	Supplemented	0.52 ± 0.04	0.34 ± 0.06*			
Malondihaldehyde (μmol/10^9 cells)	**Placebo**	3.68 ± 0.30	1.34 ± 0.17*	x		
	Supplemented	3.89 ± 0.27	1.42 ± 0.19*			

Statistical analysis: Two-way ANOVA. (T) significant effect of time (3 months). (S) significant effect of supplementation. (TxS) significant interaction between the ANOVA factors. (*) significant differences between before and after, $p < 0.05$

Discussion

Effects of training and a multivitamin and coenzyme Q_{10} supplementation on the basal status of soccer players

All the sportsmen that took part in this study, both in the placebo and supplemented group, did the same training sessions and played approximately the same matches. The soccer players took a balanced diet which provided them with all necessary nutrients and energy. Vitamin E and vitamin C intake exceeded the RDA (140% and 220 %, respectively) which is in accordance with previous studies in which sportsmen had a vitamin intake above the RDA (Cases et al., 2005; Sureda et al., 2004). Therefore, in the supplemented group the antioxidant vitamin dosage increased above the recommended dietary allowances between 3- and 4-fold.

Lymphocytes showed higher catalase activity and lower glutathione reductase activity at the beginning of the season than at the end. The Michaelis constant (Km) of catalase for hydrogen peroxide is higher than glutathione peroxidase Km, thus indicating that catalase has the ability to detoxify hydrogen peroxide when it is produced at high concentrations, while glutathione peroxidase can detoxify H_2O_2 when it is produced at a lower rate. At H_2O_2 concentrations above 10^{-5} mol/L, catalase contributes almost exclusively to the overall turnover of H_2O_2 (Mueller et al., 1997). The disposal of H_2O_2 by catalase does not involve energy expenditure or glutathione consumption, while its disposal by glutathione peroxidase consumes glutathione, which may be regenerated to its reduced form by glutathione reductase consuming NADPH. The change observed during the training period in the catalase and glutathione reductase activities could reflect a change in the strategy to eliminate ROS, from a catalase-based system to a glutathione-based system. These results are in accordance with previous studies, which detected an increase in the glutathione reductase activity after a training period in erythrocytes (Evelo et al., 1992; Ohno et al., 1988). Concerning catalase, different studies have obtained contradictory results: training has been shown to both maintain (Miyazaki et al., 2001) and increase (Ohno et al., 1988) basal values in erythrocytes. Our present results show that three months of soccer training decreased basal catalase activity in lymphocytes, thus suggesting a different regulation between lymphocyte and erythrocyte. It must be noted, however, that these previous experiments were performed with sedentary subjects, while we worked with already well-trained soccer players. The consumption of nutritional supplementation with low doses of antioxidants, vitamins and

Table 6. Work executed by the sportsmen during the match

	Placebo	Supplemented
VO_2max pre-match (ml kg^{-1} min^{-1})	57.7 ± 1.1	55.1 ± 1.2
Energy (kcal)	924 ± 34	935 ± 57
Fcmed (beats min^{-1})	163 ± 4	165 ± 5
Z3-Z5 (%)	70.0 ± 8.1	67.8 ± 10.6

Z3-Z5 indicates percentage of time spent between Zone 3 and Zone 5 during the match.

Table 7. Effects of a soccer match on the lymphocyte antioxidant enzymes after an antioxidant supplementation.

		Before match	After match	ANOVA M	S	MxS
Lymphocytes (10^6/ml)						
	Placebo	2.41 ± 0.17	2.01 ± 0.15			
	Supplemented	2.56 ± 0.32	2.37 ± 0.27			
Catalase (K/10^9 cells)						
	Placebo	5.74 ± 0.55	6.85 ± 0.69			
	Supplemented	6.42 ± 0.88	7.38 ± 0.80			
Glutathione peroxidase (nkat/10^9 cells)	**Placebo**	67.3 ± 7.6	69.3 ± 9.9			
	Supplemented	81.0 ± 16.2	68.9 ± 10.7			
Glutathione reductase (nkat/10^9 cells)	**Placebo**	59.7 ± 6.6	60.0 ± 7.0			
	Supplemented	50.0 ± 4.4	53.3 ± 6.1			
Superoxide dismutase (pkat/10^9 cells)	**Placebo**	69.7 ± 7.0	80.5 ± 6.5			
	Supplemented	87.2 ± 8.2	86.0 ± 9.1			

Statistical analysis: Two-way ANOVA (M) significant effect of the match. (S) significant effect of supplementation. (MxS) significant interaction between the ANOVA factors (*) significant diferences between before and after, $p<0,05$.

coenzyme Q, additional to a recommended nutritional intake, did not change the previously described lymphocyte antioxidant defense adaptation.

Lymphocyte MDA levels decreased about 60% during the three months of training, suggesting that lymphocytes were more protected at the end of this period than at the beginning. This protection against oxidative damage is in accordance with the increased glutathione reductase activity discussed above. The two antioxidant vitamins studied showed different responses: α-tocopherol decreased after the training period, both in the placebo and supplemented group, while ascorbate maintained its basal values. The mechanism by which lymphocytes uptake Vitamin E from plasma is as yet unknown, but the tocopherol associated protein (TAP) could participate in vitamin E transport or accumulation into cells, as occurs in liver and probably in neutrophils (Sureda et al., 2007). The decrease in α-tocopherol levels in lymphocytes during the training period could be related to the decrease in MDA, since α-tocopherol is the main molecular defense against lipid peroxides. The reduced basal oxidative stress evidenced by the low lipid peroxide levels could avoid the induction of vitamin E transport inside the lymphocyte.

H_2O_2 mitochondrial production can be due to a variety of causes. As ubiquinone within the electron transport chain cycles between the quinone to semiquinone to quinol states, there is a tendency for an electron to pass to oxygen directly (generating superoxide) instead of to the next electron carrier in the chain (Cadenas and Davies, 2000). Thus, coenzyme Q could be important in mitochondrial H_2O_2 production since it is the substrate for the first respiratory chain complex. However, in the present study, diet supplementation including ubiquinone - which has been observed to be effective to increase its plasmatic levels (Actis-Goretta et al., 2004; Watts et al., 2002) - did not affect the basal lymphocyte capability to generate hydrogen peroxide. The training period did not affect the capability to produce H_2O_2 either.

Effects of a soccer match on the oxidant and antioxidant lymphocyte status

Several studies have been carried out concerning the effects of an acute episode of physical activity such as maximal and submaximal exercise tests (Tauler et al., 2005) or a duathlon competition (Tauler et al., 2003) on the lymphocyte oxidant and antioxidant status. The effect of a soccer match had not been studied until now. We analyzed some enzymatic and non-enzymatic antioxidant defenses and oxidative damage markers in lymphocytes of sportsmen who had played a training soccer match lasting 60 minutes. Intense exercise often produces lymphopenia (Sureda et al., 2005), but in the present study there was no significant change in the lymphocyte count. Lymphocyte ROS production was maintained at basal levels after the match, but when stimulated with PMA there was an increase in the ROS production in the lymphocytes obtained after the match, thus suggesting that with the match

Table 8. Effects of a soccer match on the levels of low molecular weight antioxidants and malondialdehyde on lymphocytes.

		Before match	After match	ANOVA M	S	MxS
Ascorbate (mM)						
	Placebo	2.56 ± 0.17	3.06 ± 0.27			
	Supplemented	3.16 ± 0.43	3.58 ± 0.51			
α-tocopherol (mM)						
	Placebo	0.27 ± 0.02	0.33 ± 0.03			
	Supplemented	0.34 ± 0.06	0.32 ± 0.05			
Malondialdehyde (μmol/10^9 cells)	**Placebo**	1.34 ± 0.17	1.86 ± 0.27	x		
	Supplemented	1.42 ± 0.19	1.92 ± 0.31			

Statistical analysis: Two-way ANOVA. (M) significant effect of the match. (S) significant effect of supplementation. (MxS) significant interaction between the ANOVA factors. (*) significant diferences between before and after, $p < 0,05$.

the lymphocytes became more susceptible to produce ROS. This is in accordance with the results obtained in a previous study in which PMA-stimulated lymphocyte ROS production increased after a scuba diving session (Ferrer et al., 2007).

The antioxidant defenses, both enzymatic and non-enzymatic, remained unchanged 1 hour after the match, suggesting that the prooxidant effects of this kind of exercise observed in similar experiments (Sureda et al., 2007) is counteracted by the changes in the antioxidant strategy observed in lymphocytes in basal conditions. The match is not sufficient to induce the activation or mobilization of additional defenses. However, there was evidence of a prooxidant response induced by the match, since MDA levels were higher after the match. The supplementation did not affect any of the parameters studied and could not prevent the increased susceptibility to produce ROS and the appearance of oxidative damage in lymphocytes after the match.

In summary, regular soccer training modifies the strategy to eliminate ROS, from a catalase to a glutathione reductase based system, and increases protection against basal levels of oxidative damage markers. However, after this period of training, a training soccer match raises lymphocyte capability to produce ROS and induces oxidative damage, but it is not enough to induce a defensive response. Diet supplementation with low doses of antioxidant vitamins and coenzyme Q does not affect the lymphocyte response in well-trained sportsmen who already exceeded the RDA for the antioxidant vitamins before supplementation.

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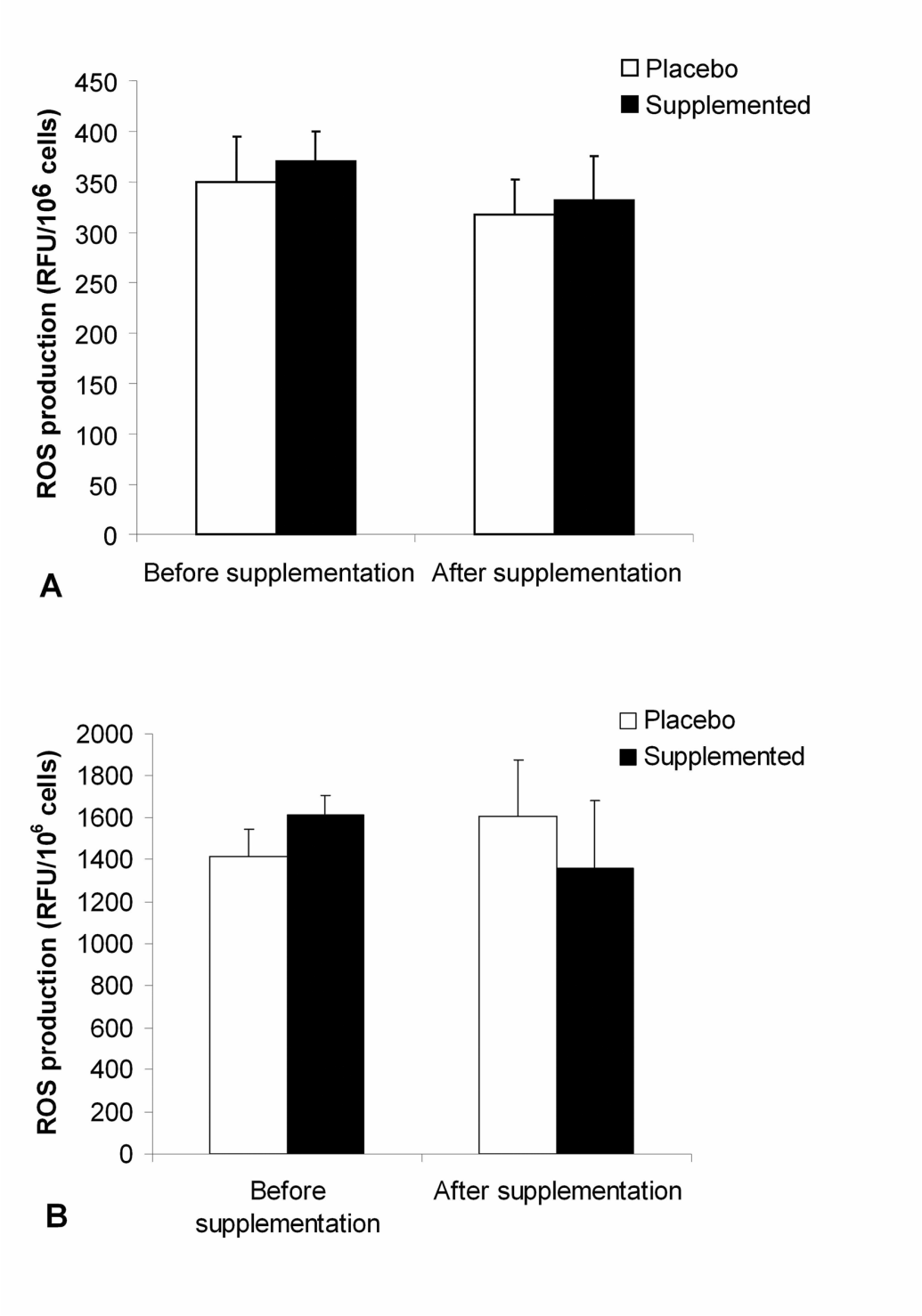
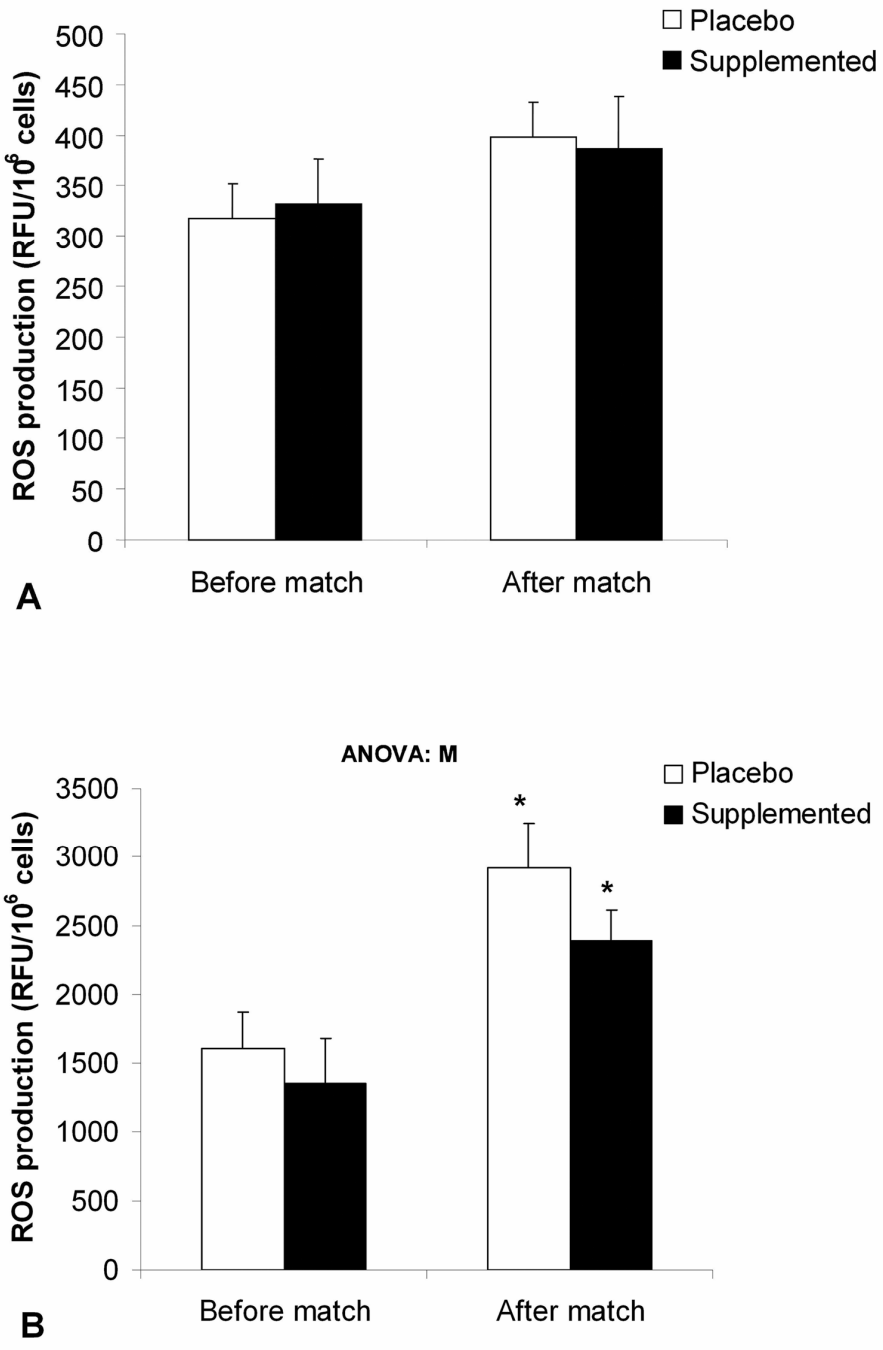
FIGURES

Figure 1. Effects of an antioxidant supplementation on the lymphocyte reactive oxygen species (ROS) production.



(A) ROS production in basal conditions. (B) ROS production after lymphocyte stimulation with phorbol myristate acetate (PMA). Statistical analysis: Two-way ANOVA. No significant differences, $p < 0.05$.

Figure 2. Effects of a soccer match on the lymphocyte reactive oxygen species (ROS) production after an antioxidant supplementation.



(A) ROS production in basal conditions. (B) ROS production after lymphocyte stimulation with phorbol myristate acetate (PMA). (M) significant effect of the match. (*) significant differences between before and after, $p < 0.05$.

Manuscript X

Enzyme antioxidant defenses and oxidative damage in red blood cells of variegate porphyria patients.

Ferrer MD, Tauler P, Sureda A, Romaguera D, Llompart I, Palacín C, Orfila J, Tur JA, Pons A.

Enzyme antioxidant defences and oxidative damage in red blood cells of variegate porphyria patients

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Abstract

Variegate porphyria (VP) is the result of decreased protoporphyrinogen oxidase (PPOX) activity, the penultimate enzyme of haem biosynthesis. Haem precursors can produce free radicals and activate oxygen inducing oxidative stress. Our aim was to analyse the effects of VP on the circulating erythrocytes, haemoglobin levels, antioxidant enzyme activities and oxidative damage. Twelve women affected by VP and twelve control healthy women participated in the study. Women affected by VP presented reduced PPOX content and δ-aminolevulinic acid dehydratase activity in erythrocytes. Haemoglobin content and mean corpuscular volume were higher in the porphyric group. Erythrocyte glutathione reductase and superoxide dismutase activities and catalase content were higher in porphyric women, although MDA levels were also higher in the erythrocytes of the porphyric group. In conclusion, the determination of PPOX could be a useful methodology to detect variegate porphyria. Despite having higher antioxidant defences, erythrocytes of porphyric women have greater oxidative damage and higher corpuscular volume, which are both indexes of a situation of higher oxidative stress.

Keywords: Variegate porphyria, antioxidants, red blood cells, oxidative damage.

Introduction

The porphyrias are a group of metabolic disorders of haem biosynthesis that result in the accumulation of porphyrins or their precursors [1, 2]. Although porphyrias are inherited diseases, there are some agents that can precipitate acute attacks and the appearance of clinical symptoms. Some of these agents are anti-epileptic drugs, barbiturates, antibiotics, menstruation, fasting, stress, alcohol and infections [3]. Variegate porphyria (VP), an autosomal dominant type of hepatic porphyria, is the result of decreased protoporphyrinogen oxidase (PPOX) activity, the penultimate enzyme of haem biosynthesis. It is characterized clinically by skin lesions and acute attacks that can occur separately or together [2]. The chronic accumulation of haem precursors in erythrocytes, liver or other tissues is responsible for the main clinical and pathological manifestations of this disease [1, 2, 4].

The cell damage induced by the haem precursors is related to their ability to produce free radicals and to activate oxygen, inducing oxidative stress. δ-aminolevulinic acid (ALA) is a metabolite which is commonly increased in porphyrias, and it has some damaging and cytotoxic effects, such as site-specific damage to ferritin protein, inducing free iron release and oxidative stress [5]. Antioxidant defences could be altered by oxidative stress as occurs in physiological situations of increased production of radical oxygen species (ROS) such as intense physical activity [6, 7], hypoxia/reoxygenation [8, 9] and scuba diving [10]. Antioxidant defences and oxidative stress have been studied in some types of porphyria, but not in VP. Decreased plasma antioxidant vitamins levels and increased oxidative damage markers have been described in porphyria cutanea tarda patients [11, 12]. In contrast, no differences have been found in the levels of antioxidant vitamins or oxidative damage markers in acute intermittent porphyria patients [13]. The lower PPOX activity in VP could compromise

haem biosynthesis and the level and function of haem proteins. The influence of this genetic alteration on the erythrocyte concentration of haemoglobin, catalase activity and other erythrocyte parameters has not been described to date. Our aim was to analyse the effects of variegate porphyria on circulating RBC, haemoglobin levels, antioxidant enzyme activities and oxidative damage markers.

We hypothesized that erythrocytes from porphyric women would be subject to oxidative stress (with increased oxidative damage markers and antioxidant enzyme activities) as a result of their exposure to damaging haem precursors. We also hypothesized that the decreased rate of haem synthesis would affect the levels and activities of haem proteins such as haemoglobin and catalase.

Materials and methods

Subjects and study design

The study was performed with twelve women affected by VP and twelve pair-matched healthy control women. All the subjects were informed of the purpose and demands of the study before giving their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the CAIB (Palma de Mallorca, Balearic Islands, Spain).

Experimental procedure

For each subject we obtained three different samples. Blood samples were taken in the same individuals and conditions on three different occasions at four monthly intervals. Venous blood samples were obtained from the antecubital vein of control and porphyric women in resting conditions after overnight fasting. These blood samples were used to purify erythrocytes following an adaptation of the method described by Boyum [14, 15].

Haematological analysis

Haematological parameters such as erythrocyte number, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH), were determined in an automatic flow cytometer analyser Technicon H2 (Bayer) VCS system. Haemoglobin concentration was determined using Drabkin reagent. This reagent oxidizes the haem group leading to the formation of methaemoglobin which reacts with potassium cyanide forming cyanmethaemoglobin, a stable pigment which can be detected spectrophotometrically at 540 nm.

Enzymatic determinations

Catalase (CAT) activity was measured in erythrocytes by the spectrophotometric method of Aebi [16]. Glutathione reductase (GRd) activity was measured in erythrocytes by a modification of the Goldberg and Spooner spectrophotometric method [17]. Glutathione peroxidase (GPx) activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler [18]. Superoxide dismutase (SOD) activity was measured in erythrocytes by an adaptation of the method of McCord & Fridovich [19]. δ-aminolevulinic acid dehydratase (ALA-DH) activity was measured following the method of Berlin & Schaller [20]. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37ºC.

MDA determination

MDA as a marker of lipid peroxidation was analyzed in 1/100 diluted erythrocytes using a colorimetric assay kit (Calbiochem, San Diego, CA, USA). The method used is specific for MDA determination.

Western blot analysis

Catalase and PPOX protein levels were determined by Western blot. Erythrocyte samples were treated with RIPA and total protein concentrations were measured by the method of Bradford [21]. Protein extracts were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For catalase 20 μg of total protein was loaded on a 12.5% agarose gel. Following electrophoresis, samples were transferred onto a nitrocellulose membrane and incubated with a primary monoclonal anti-CAT antibody (Calbiochem) and a secondary anti-Rabbit IgG peroxidase-conjugated antibody. For PPOX, 75 μg of total protein was loaded on a 12.5% agarose gel. Following electrophoresis, samples were transferred onto a nitrocellulose membrane and incubated with a primary monoclonal anti-PPOX antibody (Santa Cruz) and a secondary anti-Goat IgG peroxidase-conjugated antibody. In both cases the signal was visualized using ECL reagent (GE Healthcare) and Hyperfilm™ (Amersham Biosciences). Image analysis was performed using Kodak 1D 3.5 software.

Statistical analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 13.0 for Windows). Results are expressed as mean ± S.E.M. and $P<0.05$ was considered statistically significant. A Kolmogorov-Smirnov test was applied to assess the distribution of the data. When the data were normally distributed, the statistical significance was assessed by Student's t-test for unpaired data. When the data were not normally distributed, a Mann-Whitney test was applied to assess the statistical significance.

Results

All the subjects included in the porphyric group were clinically diagnosed to suffer from variegate porphyria. A high excretion of urinary coproporphyrins and faecal protoporphyrins is usually associated to VP although asymptomatic carriers can present normal levels of excreted porphyrins. Only two of our patients presented high levels of urinary coproporphyrins, while six of them presented high levels of faecal protoporphyrins. A plasma fluorescence emission peak at 626 nm is a characteristic feature of VP and was detected in five of the patients. 10 of the 12 patients presented clinical manifestations of porphyria such as acute attacks with severe abdominal pain, nausea, vomiting and constipation and cutaneous symptoms such as blistering, scarring and changes in pigmentation. Despite this clinical evidence we searched for an internal marker to ensure that the subjects in the porphyric group had a deficiency in the machinery to synthesize the haem group. PPOX levels were determined by western blot and the relative content of this protein is shown in Figure 1B. PPOX content was significantly reduced in the porphyric groups to less than 50% of controls. 80% of the porphyric patients presented a relative PPOX content below the lower limit of their controls.

Table I. Effect of variegate porphyria disease on erythrocyte characteristics and oxidative damage.

	Control	Porphyria
RBC (10^6/μl)	4.55 ± 0.05	4.56 ± 0.04
Haemoglobin (g/dl)	13.0 ± 0.2	13.6 ± 0.1 #
Haematocrit (%)	39.0 ± 0.7	40.2 ± 0.6 #
Mean corpuscular volume (fl)	84.5 ± 1.4	88.2 ± 0.5 #
Mean corpuscular haemoglobin (pg)	28.6 ± 0.3	29.8 ± 0.3 #
MDA (μmol/10^{12} cell)	17.9 ± 2.5	25.1 ± 2.1 #

Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups, $p < 0.05$.

Table II. Effect of variegate porphyria disease and age on erythrocyte antioxidant enzyme activities.

	Control	Porphyria
Catalase (K/10^9 cell)	5.20 ± 0.19	5.54 ± 0.24
GPx (nkat/10^9 cell)	13.2 ± 0.5	12.9 ± 0.4
GRd (nkat/10^9 cell)	5.68 ± 0.31	8.20 ± 0.96 #
SOD (pkat/10^9 cell)	2.86 ± 0.21	3.43 ± 0.18 #

Statistical analysis: For catalase and GPx a Student's t-test for unpaired data was applied. For GRd and SOD a Mann-Whitney test was applied. (#) indicates significant differences between porphyria and control groups, $p<0.05$.

VP significantly affected ALA-DH activity in erythrocytes of the patients studied (Fig. 1C). Porphyric women presented about 57% of the ALA-DH activity of control women. The RBC counts were not affected by VP (Table I). Blood haemoglobin, hematocrit, MCV and MCH in porphyria women was significantly higher than their controls. Oxidative damage was assessed by measuring MDA content in erythrocytes, and porphyric women presented significantly higher MDA content in erythrocytes than their controls (Table I).

The antioxidant enzyme activities in erythrocytes of controls and porphyric women are shown in Table II. Catalase activity was similar in all groups, but catalase levels (Fig. 2) were significantly affected by the porphyric condition. Porphyric women presented significantly higher catalase levels than their respective controls. Glutathione peroxidase (GPx) activity was not affected by the disease. Glutathione reductase (GRd) and superoxide dismutase (SOD) activities were affected by porphyria, being higher in porphyric women than in controls.

Discussion

Variegate porphyria is the result of decreased PPOX activity, the penultimate enzyme of haem biosynthesis. This disease is characterized by

accumulation of some haem precursors in erythrocytes and by a low rate of haem biosynthesis as a result of the genetic error in PPOX. The diagnosis of VP is difficult and to date no methodology has been shown to be 100% reliable for the detection of the disease. All the patients participating in our study were clinically diagnosed on a basis of different parameters such as plasma fluorescence peak at 626 nm, levels of excreted urinary and faecal porphyrins and clinical manifestations. In addition we measured PPOX protein levels in total blood and found that the 80% of the porphyric patients presented a relative PPOX content below the lower limit of their controls, suggesting that the determination of PPOX levels could be a useful methodology to detect variegate porphyria, although it is not fully conclusive because we have not analyzed PPOX content in other types of porphyria, so we can not know whether PPOX protein levels are altered in other porphyrias. Although decreases in PPOX activity have been previously reported as a result of variegate porphyria, decreased PPOX protein levels have not been described until now. We propose PPOX protein level determination as an easy way to provide additional evidence of the disease, although we do not show it as a conclusive or exclusive marker for variegate porphyria. The accumulation of haem precursors such as δ-aminolevulinic acid (ALA) has been shown to induce the oxidation of haem biosynthesis enzymes, especially ALA-dehydratase (ALA-DH) [22]. In our study erythrocytes from porphyric women presented lower ALA-DH activity than controls. This could be attributed to increased oxidative damage to ALA-DH protein and reaffirms their condition of porphyric in addition to their clinical diagnosis.

The porphyric condition did not affect the red cell counts, which is in accordance with a similar pattern of erythrocyte generation in both porphyric and control women. However the mean corpuscular volume of VP women is significantly higher than controls. Oxidative damage of RBC membranes frequently produces a rise in the cell volume, a decrease in osmotic resistance and haemolysis [23-25]. The increased corpuscular volume in porphyria patients is accompanied by increased MDA content, which is an index of lipid peroxidation and also indicates greater oxidative damage in the RBC of porphyric women than controls. VP induces slightly higher blood haemoglobin, greater mean corpuscular haemoglobin and a higher hematocrit. The method we used to determine haemoglobin content ensures that the protein measured also contains the haem group. Therefore, the low rate of haem production in VP is enough to generate the same - or even greater - quantity of haemoglobin as control women. It has been pointed out that haemoglobin may function in the erythrocytes in the protection against oxidative damage by acting as an oxidant sink in protecting the membrane from radical attack [26]. However, haemoglobin can also be converted to methaemoglobin and superoxide anion, and reduced back by the methaemoglobin reductase system [27]. The high haemoglobin concentration in porphyric women could contribute to increasing their superoxide anion production. A high production of free radicals can affect the endogenous antioxidant enzyme defences, in accordance with the role of ROS such as hydrogen peroxide in activating the synthesis of antioxidant enzymes such as superoxide dismutase [28, 29]. In this instance, the antioxidant enzymes glutathione reductase and superoxide dismutase are increased in the porphyric group. Glutathione peroxidase and catalase activities are unaffected by variegate porphyria; catalase protein levels, however, are higher in porphyric than control women. These results suggest that the limitations in free haem biosynthesis could influence the availability of active catalase for RBC even though the enzyme is over-expressed. An inactivation of catalase could also explain this lower specific activity of catalase in the porphyric women, although we found no evidence of inactivation of other antioxidant enzymes such as glutathione reductase and superoxide dismutase.

Our results show that PPOX protein levels are reduced in variegate porphyria patients and the determination of PPOX could be a useful methodology to detect VP. Furthermore, VP accentuates the oxidative stress situation in RBC. Despite having higher antioxidant defences, erythrocytes of porphyric women display greater oxidative damage and higher corpuscular volume, which are both indexes of a situation of higher oxidative stress. Catalase protein content is increased in porphyric women while catalase activity is maintained, suggesting an impairment of the enzyme as a consequence of limited haem synthesis or specific catalase inactivation.

Acknowledgements

This work has been granted by the Spanish Ministry of Science and Education (DEP2005-00238-C04-02/EQU and AGL2007-62806/ALI) and FEDER founds. MD Ferrer was funded by grant of the Spanish Ministry of Science and Education. We are thankful to Ms Magdalena Ordinas, Asociación Española de Porfíria, Delegació de les Illes Balears, who collaborated in the family studies.

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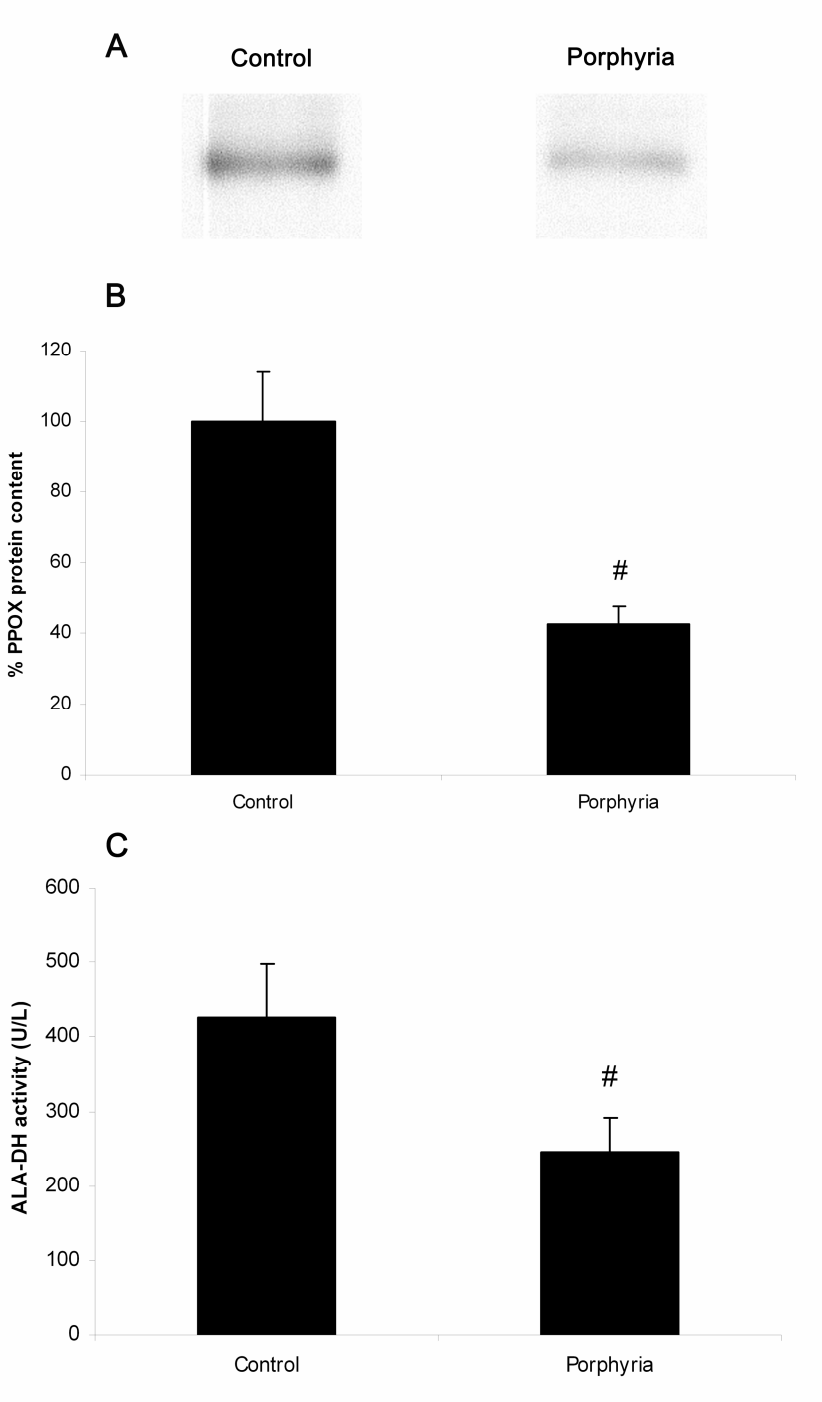
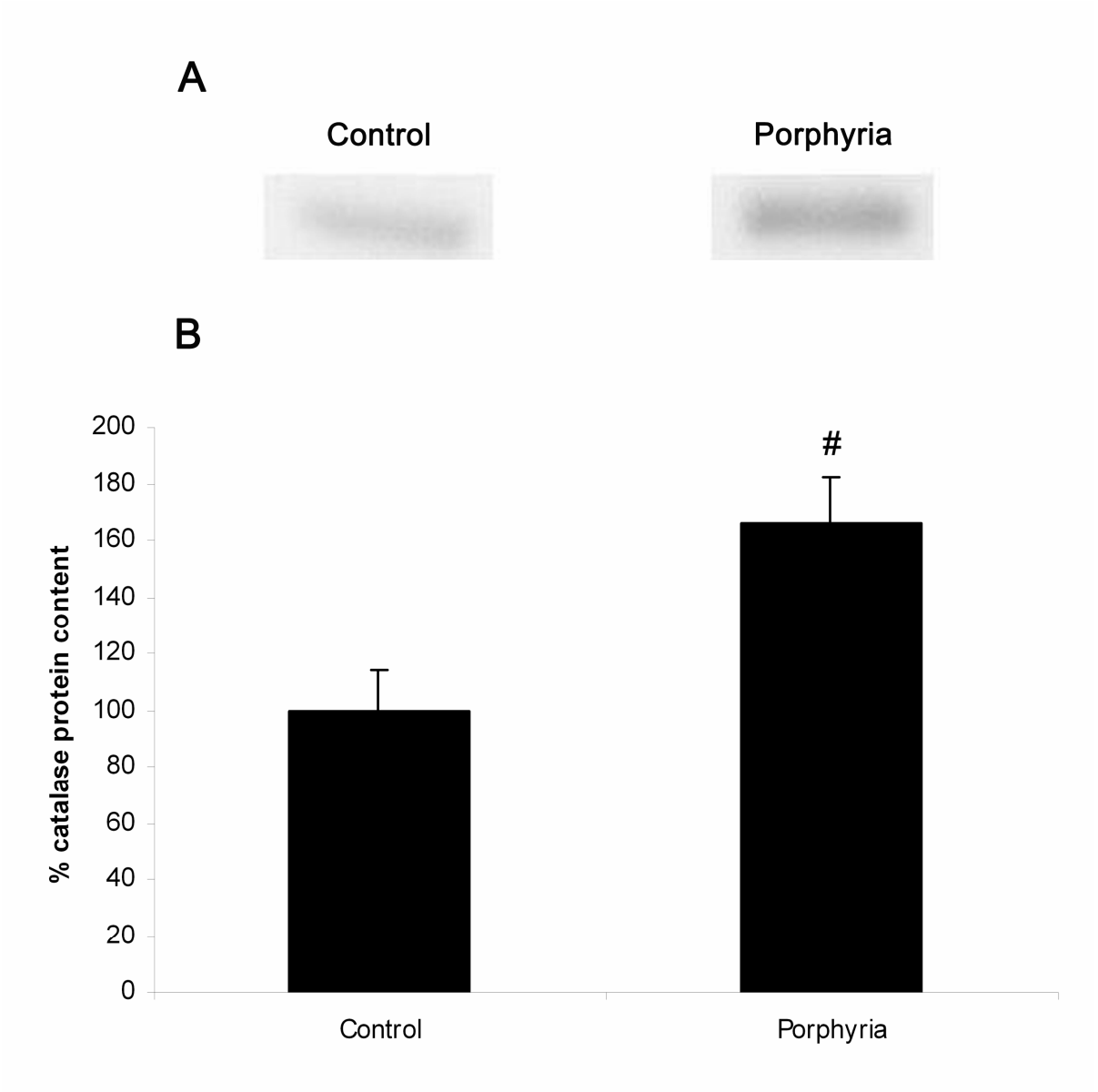
FIGURES

Figure 1. Effects of variegate porphyria on PPOX protein content and δ-aminolevulinic acid dehydratase activity in erythrocytes.



The study was performed with 12 women affected by variegate porphyria and 12 healthy control women. Three different samples were collected from each subject at 4 monthly intervals. 75 μg of total erythrocyte protein was loaded to determine PPOX content by western blot. (A) Western blot images of representative samples for each studied group. (B) Relative PPOX protein content. Total PPOX content in the control group was normalized to 100%. (C) ALA-DH activity (U/L) in erythrocytes. Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups ($p < 0.05$).

Figure 2. Effects of variegate porphyria on catalase protein content



The study was performed with 12 women affected by variegate porphyria and 12 healthy control women. Three different samples were collected from each subject at 4 monthly intervals. 20 μg of total erythrocyte protein was loaded to determine catalase content by western blot. (A) Western blot images of representative samples for each studied group. (B) Relative catalase protein content. Total catalase content in the control group was normalized to 100%. Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups ($p < 0.05$).

Manuscript XI

Impaired mitochondrial antioxidant defences in variegate porphyria are accompanied with more inducible ROS production and oxidative damage.

Ferrer MD, Tauler P, Sureda A, Palacín C, Tur JA, Pons A.

Impaired mitochondrial antioxidant defences in Variegate Porphyria are accompanied by more inducible ROS production and oxidative damage

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Abstract
Variegate porphyria (VP) is the result of decreased protoporphyrinogen oxidase (PPOX) activity, the penultimate enzyme of heme biosynthesis. Heme precursors can produce reactive oxygen species (ROS) inducing oxidative stress. Our aims were to analyse the influence of VP on lymphocyte ROS production and detoxification mechanisms and on the appearance of oxidative damage. Twelve women affected by VP and twelve pair matched healthy control women participated in the study. Porphyric women presented impaired expression of the mitochondrial proteins PPOX, UCP-3, Bcl-2 and sirtuin 3. Lymphocytes from porphyric women presented higher H_2O_2 production than control women after stimulation with PMA. The inhibition of H_2O_2 production after treatment with myxothiazol pointed towards complex III of the mitochondrial respiratory chain as the main contributor of the higher ROS production in porphyric subjects. No differences were observed between porphyric and control subjects in the basal levels of DNA damage. However, DNA damage expressed both as a percentage of DNA in tail and as the tail moment, was greater in porphyric women than controls after lymphocyte treatment with H_2O_2. In conclusion, lymphocytes from variegate porphyria women showed impaired expression of mitochondrial antioxidant defenses but no significant signs of oxidative stress were evidenced in basal conditions; however, lymphocytes of porphyric women were more susceptible to producing mitochondrial reactive oxygen species and to suffering oxidative damage when submitted to stressful situations.

Introduction

Heme is a prosthetic group present in heme proteins such as hemoglobin or myoglobin, mitochondrial or microsomal cytochromes, catalase and peroxidases, all of which are proteins playing very important roles in cell function [1]. The porphyrias are a group of metabolic disorders of heme biosynthesis that result in the accumulation of porphyrins or their precursors [2, 3]. Although porphyrias are inherited diseases, there are some agents that can precipitate acute attacks and the appearance of clinical symptoms [4]. Variegate porphyria (VP), an autosomal dominant type of hepatic porphyria, is the result of decreased protoporphyrinogen oxidase (PPOX) activity, the penultimate enzyme of heme biosynthesis. It is characterized clinically by skin lesions and acute attacks that can occur separately or together [3]. The chronic accumulation of heme precursors in erythrocytes, liver or other tissues is responsible for the main clinical and pathological manifestations of this disease [2, 3, 5]. The accumulation of heme precursors in other cell types can induce cellular damage due to their ability to produce free radicals and to activate oxygen, inducing oxidative stress. However, other mechanisms could be involved in the generation of greater amounts of reactive oxygen species in porphyric patients. The mitochondrial electron transport chain is one of the main sources of intracellular reactive oxygen species (ROS) in many cell types. Intermediate free radical species of mitochondrial electron-transporters may interact with dissolved oxygen converting it into superoxide anions [6]. The sites of superoxide generation are located in both complex I [7, 8] and complex III. Defects inducing a decrease in electron transfer in the respiratory chain lead to enhanced ROS production. As heme is an essential factor present in several cytochromes belonging to complexes III and IV, the dysfunction in heme biosynthesis could lead to a disturbed function of the respiratory chain, thus leading to increased ROS production. Although H_2O_2 sources in the lymphocyte are not completely known, some other possible sources are nitric oxide synthase (NOS), xanthine oxidase (XOD), NADPH oxidase and glucose oxidase (GOD) enzymes. Moreover, when lymphocytes are stimulated with

phorbol myristate acetate (PMA) ROS production increases [9, 10] by an unknown mechanism in which the PKC signaling pathway seems to be activated [11-13].

Antioxidant defences in lymphocytes can be altered by oxidative stress in physiological situations of increased production of reactive oxygen species (ROS) such as intense physical activity [14, 15], hypoxia/reoxygenation [16, 17] and scuba diving [9]. Antioxidant defences and oxidative stress have been studied in some types of porphyria, but not in VP. Decreased plasma antioxidant vitamin levels and increased oxidative damage markers have been described in porphyria cutanea tarda patients [18, 19]. In contrast, no differences have been found in the levels of antioxidant vitamins or oxidative damage markers in acute intermittent porphyria patients [20]. The lower PPOX activity in the lymphocytes of variegate porphyria patients could compromise heme biosynthesis and the level and function of heme proteins. In addition, some mitochondrial proteins have been related to the regulation of ROS production by the electron transport chain. UCP-3 is a member of the mitochondrial uncoupling protein family and has been shown to act as an antioxidant by reducing reactive oxygen species production [21]. Bcl-2 is localized in the outer mitochondrial membrane and could be important in the appearance of mitochondrial reactive oxygen species and oxidative damage [22]. SIRT3, a mitochondrial member of the sirtuin family of histone deacetylases [23], is known to play an important role over ROS production and scavenging in mitochondria by decreasing mitochondrial membrane potential and increasing cellular respiration [24].

Our aim was to evaluate the role of mitochondria in lymphocyte ROS production, the expression of mitochondrial antioxidant defences and the oxidative damage related to Variegate Porphyria. We hypothesized that lymphocytes from variegate porphyria patients would show higher mitochondrial ROS production and oxidative damage markers and impaired mitochondrial antioxidant defences when compared to healthy women.

Materials and methods

Subjects and study design

The study was performed with twelve women affected by VP and twelve pair matched healthy control women. All the subjects were informed of the purpose and demands of the study before giving their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the CAIB (Palma de Mallorca, Balearic Islands, Spain). All the patients participating in our study had been previously diagnosed to suffer from variegate porphyria on the basis of different parameters such as plasma fluorescence peak at 626 nm, levels of excreted urinary and fecal porphyrins and clinical manifestations such as abdominal pain during porphyric attacks.

Venous blood samples were obtained from the antecubital vein of control and porphyric women in resting conditions after overnight fasting. The lymphocyte fraction was purified from whole blood following an adaptation of the method described by Boyum [9, 25].

mRNA gene expression

mRNA expressions were determined by real time RT-PCR with 18S ribosomal as a reference gene. For this purpose, mRNA was isolated from lymphocytes by phenol-chloroform extraction. cDNA was synthesized from 1 μg total RNA using reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. The primers used are shown in Table 1. For all PCRs there was one cycle at 95ºC for 10 min, followed by 40 cycles at the conditions shown in Table 1.

The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Basal mRNA levels at the beginning of the stage were arbitrarily referred to as 1. The expression of the target gene was normalized with respect to ribosomal 18S.

Hydrogen peroxide production

H_2O_2 production in lymphocytes was measured before and after stimulation with phorbol myristate acetate (PMA) using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as an indicator. DCFH-DA (30 μg/ml) in PBS was added to a 96-well microplate containing 50 μl lymphocyte suspension. PMA (3 μM) prepared in HBSS or HBSS alone was added to the wells and the fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37ºC for 1 hour in a FLx800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc.).

Table 1. Primers and conditions used in Real Time PCRs

Gene	Primers	Conditions
18S	Fw: 5'-ATG TGA AGT CAC TGT GCC AG-3'	95ºC,10 s
	Rv: 5'-GTG TAA TCC GTC TCC ACA GA-3'	60ºC, 7 s
		72ºC, 12 s
PPOX	Fw: 5'-TTT GGC TAC TTT GAG GTC AC-3'	95ºC, 10 s
	Rv: 5'-TCC CCA TTT GCA TTA ACC AG-3'	60ºC, 10 s
		72ºC, 15 s
UCP-3	Fw: 5'- CGT GGT GAT GTT CAT AAC CTA TG-3'	95°C, 5 s
	Rv: 5'- CGG TGA TTC CCG TAA CAT CTG-3'	60ºC, 7 s
		72ºC, 10 s
Bcl-2	Fw: 5'-CTG GTG GGA GCT TGC ATC AC-3'	95°C, 5 s
	Rv: 5'-ACA GCC TGC AGC TTT GTT TC-3'	65ºC, 5 s
		72ºC, 5 s
SIRT3	Fw: 5'-GAG CTT CTG GGC TGG ACA GA-3'	95°C, 10 s
	Rv: 5'-TGG GAT GTG GAT GTC TCC TAT G-3'	65ºC, 5 s
		72ºC, 7 s

In addition, three treatments with 100 mM allopurinol (xanthine oxidase inhibitor), 5 mM rotenone (complex I inhibitor) or 2 mM myxothiazol (complex III inhibitor) were performed in PMA-stimulated lymphocytes. All incubations were performed in the presence of digitonin to ensure internalization of inhibitors.

Comet Assay

Assessment of DNA damage was carried out using the comet assay method. Briefly, slides were prepared by adding purified lymphocytes, mixed with 0.6% low-melting-point agarose. In order to release the DNA, cells were lysed by immersing slides in lysing solution (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mN Tris, 1% Triton X-100 and 10% DMSO, pH 10) at 4ºC for 40 min. Electrophoresis was carried out with a 250 mV electric current applied for 30 min. Tris buffer pH 7.5 was added onto the slides to neutralize excess alkali. DNA was stained by adding ethidium bromide. Comet measurements were made by image analysis using a fluorescence microscope and the Comet software (TriTek CometScore™). Images of 50 random nuclei were analyzed for each sample. The comet measurements which were recorded and subsequently used for analysis were percentage DNA in tail (tail intensity) and tail moment (tail intensity x tail length). DNA damage was studied in all samples in basal conditions and after treatment with hydrogen peroxide (10 μM) for 5 min.

Statistical analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 13.0 for Windows). Results are expressed as mean ± s.e.m. and $P<0.05$ was considered statistically significant. The statistical significance of the data was assessed by Student's t-test for unpaired data.

Results

No significant differences were observed in anthropometric data between the porphyric patients and controls participating in the study. Controls presented the same age and similar life style habits as porphyric patients (data not shown) [26].

Lymphocyte H_2O_2 production is shown in Figure 1. In basal conditions, H_2O_2 production was similar in control and porphyric women. However, when lymphocytes were stimulated with PMA, H_2O_2 production rose and this was significantly higher in the porphyric group when compared to controls. The lymphocytes from variegate porphyria women were then more susceptible to producing ROS than controls when activated. The possible source of the production of H_2O_2 by lymphocytes was studied using different inhibitors of target candidates to produce ROS. Three different inhibitors were used to determine the possible source of the increased ROS production after lymphocyte stimulation with PMA. Myxothiazol, as an inhibitor of respiratory chain complex III, returned ROS production back to basal levels in both groups and the difference between porphyrics and controls found in the absence of myxothiazol disappeared. Rotenone, as an inhibitor of

respiratory chain complex I, induced a rise in H_2O_2 production in the control group but not in the porphyric group, and no differences between the two groups were found. Allopurinol, as an inhibitor of xanthine oxidase, did not modify H_2O_2 production, but no differences between porphyric and control women were detected in the presence of this inhibitor. The PMA activation of lymphocytes induced an increase in H_2O_2 production mainly due to mitochondrial production, and lymphocytes from porphyric women produced greater mitochondrial H_2O_2 amounts than controls. This mitochondrial H_2O_2 overproduction in porphyric women could be related with lower antioxidant defenses, in addition to other causes. The gene expression of some key antioxidant proteins in mitochondrial H_2O_2 production was analyzed. Figure 2 shows the gene expression of PPOX, UCP-3, Bcl-2 and SIRT3 genes in lymphocytes of control and porphyric women. PPOX (Fig. 2A) and UCP-3 (Fig. 2B) gene expressions were decreased in porphyric patients to 80% of the expression in controls. Bcl-2 (Fig. 2C) and SIRT3 (Fig. 2D) expressions were also reduced in the porphyric group when compared to controls (70% and 65% respectively). The lymphocytes from women affected by variegate porphyria had lower rates of expression of the enzyme that define their pathology (PPOX), but also in other key antioxidant proteins such as UCP-3, Bcl-2 and SIRT3, even to a greater extent than PPOX.

The imbalance between lymphocyte H_2O_2 production and antioxidant gene expression could increase oxidative damage. The levels of DNA damage in basal conditions were similar in controls and porphyric women with no significant differences between groups (Figure 3). However, after treating lymphocytes with H_2O_2, the degree of DNA damage, expressed both as a percentage of DNA in tail and as the tail moment, was significantly increased in women affected by variegate porphyria compared to controls.

Discussion

Variegate porphyria is the result of decreased PPOX activity, the penultimate enzyme of heme biosynthesis. This disease is characterized by the accumulation of some heme precursors in erythrocytes and by a low rate of heme biosynthesis as a result of the genetic error in PPOX. All the patients participating in our study had been previously diagnosed on the basis of different parameters such as plasma fluorescence peak at 626 nm, levels of excreted urinary and fecal porphyrins and clinical manifestations. We first investigated whether the PPOX expression is impaired in lymphocytes, in which the demand and utilization of heme is not as high as in the erythrocytes. We found a down-regulation (80%) of PPOX gene expression in the lymphocytes of porphyric women when compared to controls which makes it evident that the genetic problem in the PPOX gene has phenotypic consequences like decreased PPOX expression in white blood cells such as lymphocytes. The malfunction of PPOX in lymphocytes could compromise the heme synthetic pathway and hence alter the oxidative status by impairing the antioxidant systems and/or increasing ROS production.

The accumulation of the heme precursors has been shown to constitute an endogenous source of reactive oxygen species, triggering oxidative damage to cellular components [27, 28]. Therefore, we studied H_2O_2 production in lymphocytes as an indicator of intracellular ROS formation. In basal conditions, lymphocytes from porphyric and control women produced approximately the same amount of H_2O_2. However, when lymphocytes were activated with PMA, greater H_2O_2 production was detected in the porphyric group when compared to controls, indicating that after activation lymphocytes from porphyric women are more susceptible to producing ROS and hence to suffering oxidative damage. Lymphocyte ROS sources are the mitochondrial respiratory chain, nitric oxide synthase (NOS) activity in the lack of arginine, xanthine oxidase (XOD) and glucose oxidase (GOD) activities. The rate of increase in the H_2O_2 production when lymphocytes are activated with PMA in a medium lacking xanthine, and with no changes in arginine availability, as well as iNOS, XOD or GOD, '*de novo'* synthesis seems unlikely to occur, suggests that this increase could be mitochondrial. In order to demonstrate that the increased H_2O_2 production in activated lymphocytes from porphyric women was due to mitochondrial respiratory chain impairment, we tested the effects of three different inhibitors of ROS production such as myxothiazol (complex III inhibitor), rotenone (complex I inhibitor) and allopurinol (xanthine oxidase inhibitor). The allopurinol treatment did not modify H_2O_2 production in lymphocytes, thus ruling out the possibility of a xanthine oxidase activity derived increase in ROS production in variegate porphyric women. The inhibition of the mitochondrial respiratory chain with different inhibitors induced different changes. The inhibition of complex I with rotenone has been shown to induce both an increase [29] and a decrease [30] in ROS production. It

would seem that the effects of rotenone depend on whether the primary site of superoxide generation in the mitochondrial electron transport chain is complex I [31] or complex III [32], thus increasing or decreasing ROS production, respectively. No effect of complex I inhibition with rotenone was found in the porphyric patients. In the control group, however, an increase in ROS production was detected after inhibition with rotenone while no differences in H_2O_2 production were found between porphyric and control groups. This may indicate that in porphyric patients complex III could play an important role in producing more ROS than in healthy people. To further demonstrate this hypothesis, the effects of the complex III inhibitor, myxothiazol, were tested. Myxothiazol inhibits the binding of ubiquinol at complex III, thereby preventing the oxidation of ubiquinol and the formation of ubisemiquinone, and reducing ROS production [32, 33]. In our study, when complex III was inhibited with myxothiazol, decreased H_2O_2 production was detected and no differences were found between porphyric and control women. This reaffirms that the increase in H_2O_2 production in lymphocytes after stimulation with PMA and the increased production in porphyric women when compared to controls are due to the mitochondrial respiratory chain function, and mostly due to respiratory complex III, rather than to other sources such as xanthine oxidase activity.

Once evidenced the increased susceptibility of porphyric patients to producing mitochondrial ROS, we studied the expression of some proteins which have been related to the regulation of ROS by the electron transport chain. UCP-3 is a member of the mitochondrial uncoupling protein family mainly detected in skeletal muscle; it is not involved in thermogenesis [34] but has been shown to act as an antioxidant by reducing reactive oxygen species production [21]. In our study, women affected by variegate porphyria presented down-regulated UCP-3 expression in lymphocytes when compared to control women. In a previous study we evidenced that UCP-3 expression after a single bout of exercise is directly correlated to Bcl-2 expression, thus supporting the idea that both proteins could have related functions in protecting mitochondrial stability by acting as antioxidants [35]. Bcl-2 is localized in the outer mitochondrial membrane and could be important in the regulation of proton flux in mitochondria, mitochondrial membrane potential ($\Delta\Psi_M$) and the appearance of mitochondrial reactive oxygen species and oxidative damage [22]. When we measured Bcl-2 expression we found that lymphocytes from porphyric women presented lower Bcl-2 expression than controls. The decreased gene expression of both antioxidant proteins UCP-3 and Bcl-2 indicates that the antioxidant capabilities of the lymphocytes from porphyric patients are impaired, and thus they could be more susceptible to suffering oxidative stress.

SIRT3 is a member of the sirtuin family of histone deacetylases. Of the seven known sirtuins in mammals, three of them - including SIRT3 - are located in the mitochondria [23]. It has been postulated that SIRT3 activity can up-regulate the expression of mitochondrial biogenesis genes such as PGC-1α [24], but the pathways involved in these up-regulations are unclear [24]. SIRT3 has been shown to decrease mitochondrial membrane potential and reactive oxygen species production, while increasing cellular respiration [24]. The mechanism by which SIRT3 increases respiration is not completely understood as yet, but recent evidence point towards deacetylation and activation of the mitochondrial enzymes involved in the respiratory metabolism and the control of the citric acid cycle, such as isocitrate dehydrogenase – which produces NADPH, important for the NADPH-dependent regeneration of antioxidants – [36], glutamate dehydrogenase [36] and acetyl coenzyme A synthetase 2 [37]. Therefore SIRT3 clearly plays an important role in ROS production and scavenging in mitochondria. In our study, SIRT3 expression in lymphocytes of porphyric women was 65% of the expression in the control group. The combined down-regulation of UCP3, Bcl-2 and SIRT3 – all of them genes encoding proteins directly or indirectly related to mitochondrial ROS formation and detoxification – in porphyric women is indicative of impaired antioxidant defenses in these patients.

Taking into account these results, lymphocytes from porphyric women have been shown to present impaired mitochondrial antioxidant potential, but in basal conditions this impairment is not related to increased ROS production. However, when lymphocytes are activated and then exposed to a stress condition, lymphocytes from porphyric women produce greater amounts of mitochondrial ROS than control women. The combination of both impaired antioxidant defenses and increased ROS production could lead to a situation of oxidative stress, thus we measured DNA damage as an indicator of cellular damage. The comet assay is a sensitive method for detecting DNA damage at the level of individual cells, including detection of single and double strand breaks, incomplete excision repair sites and cross links. It has been

proved to be a good marker of oxidative damage in the DNA of lymphocytes [38-40]. Tail moment represents both the amount of DNA migrated into the tail and the distance migrated, and it has been reported as a valid marker of single-strand DNA breakage [41] while at low levels of damage the tail length seems to be a more sensitive marker than percentage DNA in tail [42]. Once again we found no evidence of DNA damage in the lymphocytes of porphyric women when compared to controls in basal conditions. However, after a short treatment of isolated lymphocytes with 10 mM H_2O_2, greater DNA damage was evidenced in porphyric women expressed both as % DNA in tail and tail moment. The increased oxidative damage after oxidative treatment suggests that variegate porphyria patients have difficulties to counteract the excess of reactive oxygen species produced as a consequence of a stressful situation and are hence more susceptible to suffering oxidative stress.

In conclusion, in basal conditions, lymphocytes from variegate porphyria patients showed impaired mitochondrial antioxidant defenses but no significant signs of oxidative stress were evidenced; however, lymphocytes of patients were more susceptible to producing mitochondrial reactive oxygen species and to suffering oxidative damage when submitted to stressful situations such as lymphocyte activation and H_2O_2 treatment, respectively.

Acknowledgements

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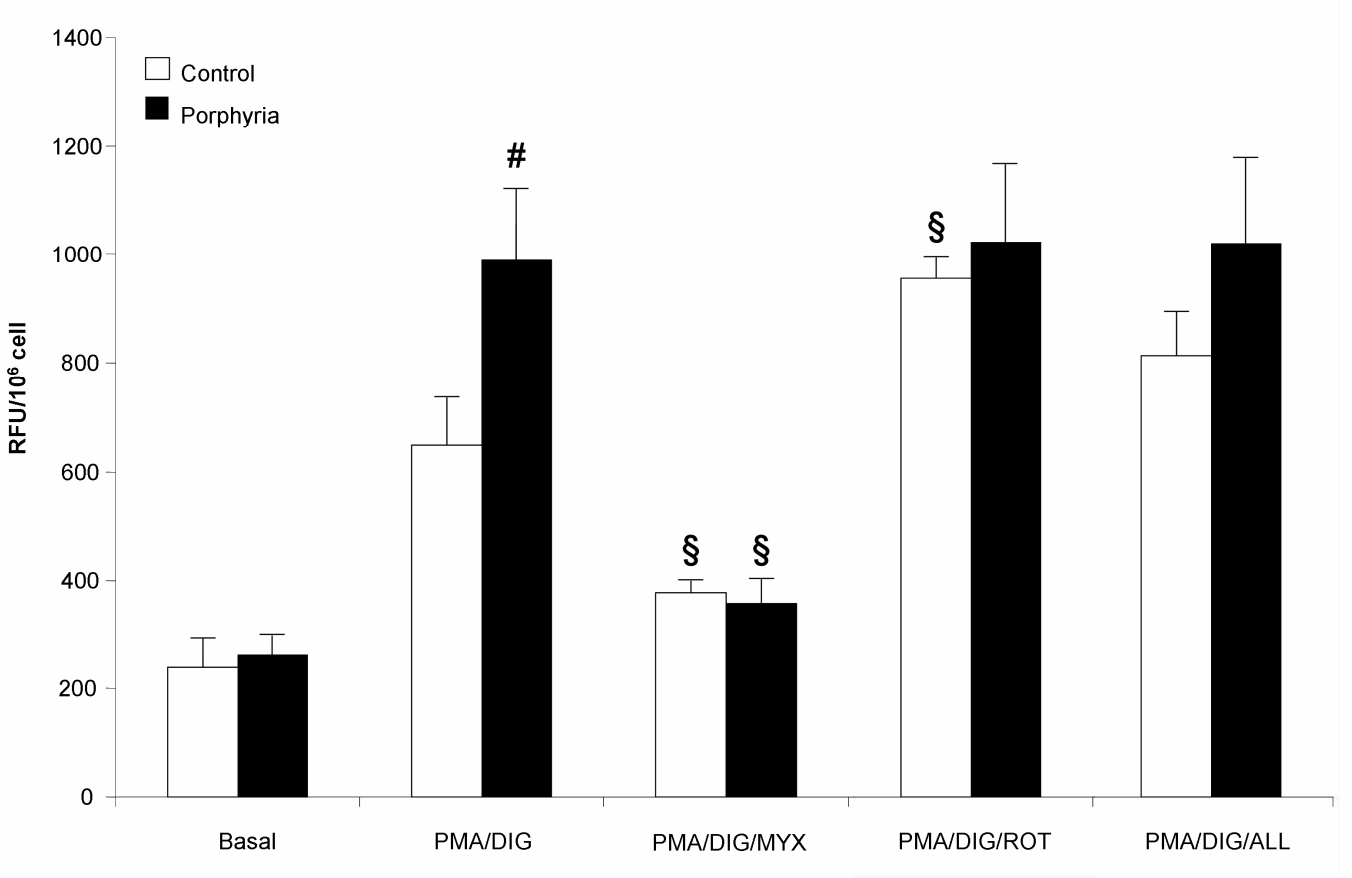
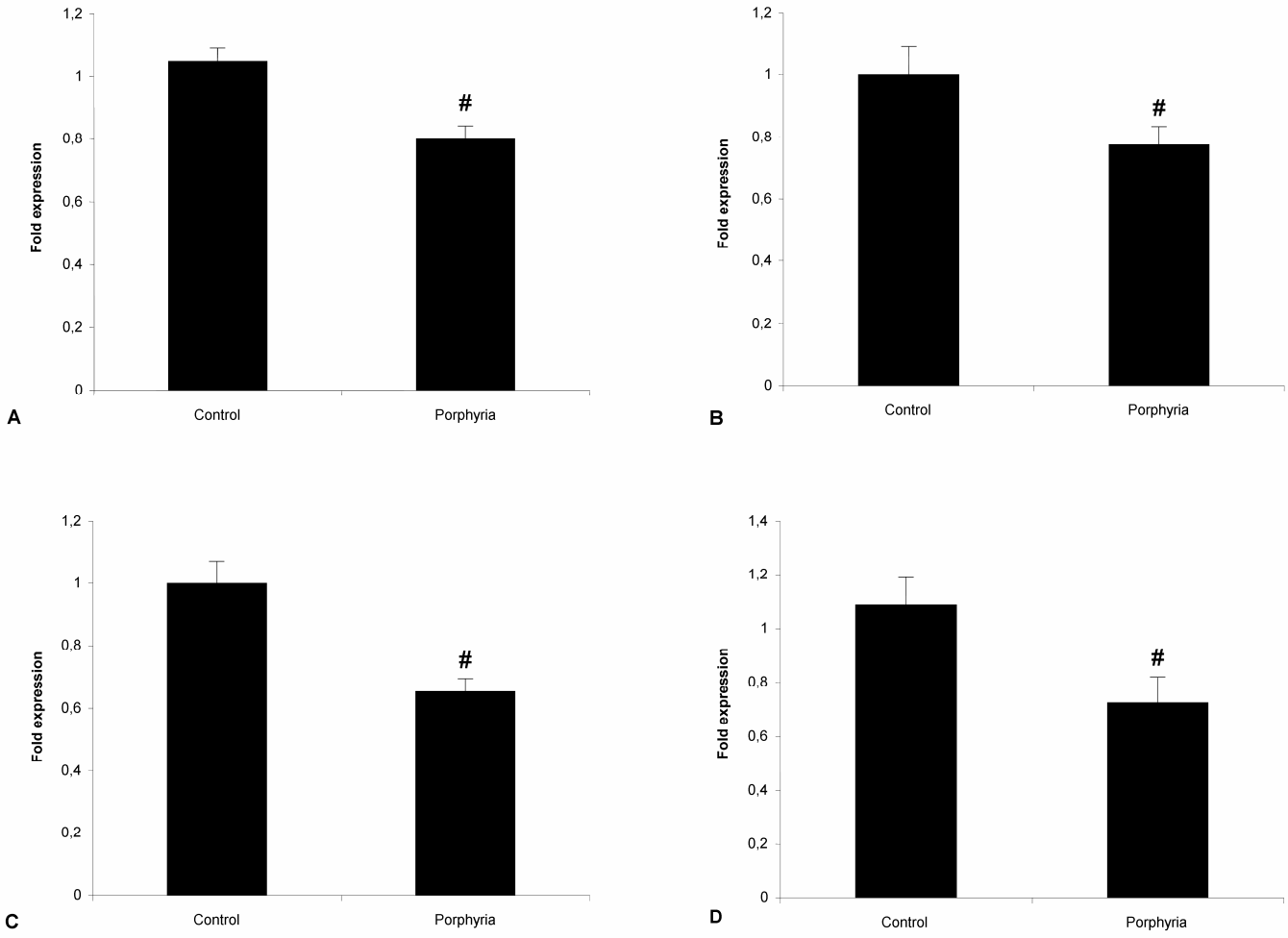
FIGURES

Figure 1. Effects of variegate porphyria on lymphocyte H_2O_2 production after addition of different inhibitors.



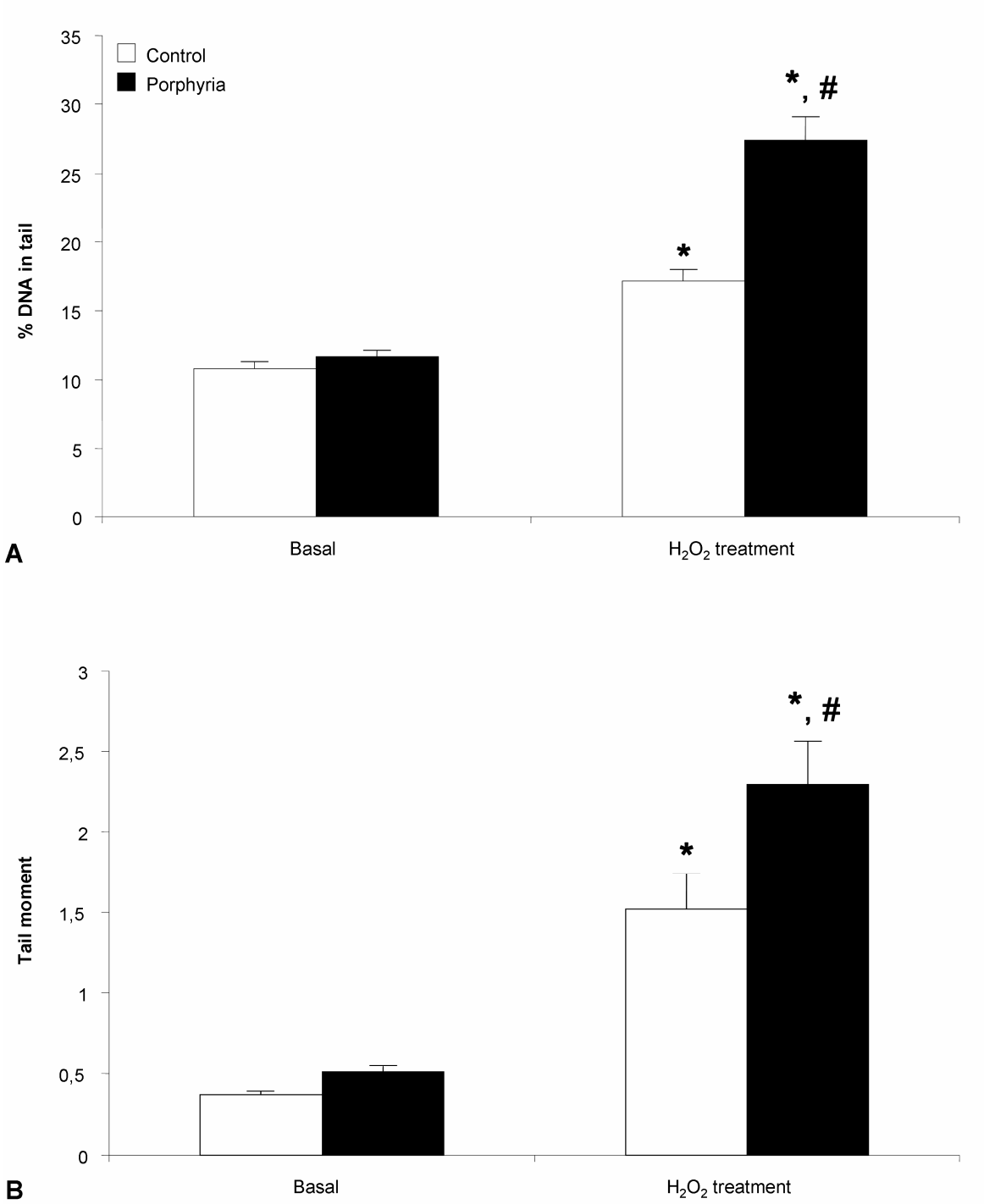
All incubations were performed in the presence of digitonin (DIG). Phorbol meristate acetate (PMA) was used to activate lymphocytes. Inhibitors tested were 2 mM myxothiazol (MYX), 5 mM rotenone (ROT) and 100 mM allopurinol (ALL). Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups, (§) indicates significant differences vs. PMA/DIG alone treatment ($p < 0.05$).

Figure 2. Effects of variegate porphyria on lymphocyte PPOX (A), UCP-3 (B), Bcl-2 (C), and SIRT3 (D) gene expression.



The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups ($p < 0.05$).

Figure 3. Effects of variegate porphyria on lymphocyte DNA damage.



% DNA in tail (A) and tail moment (B) in lymphocytes measured in basal conditions and after H_2O_2 treatment. Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between controls and porphyric patients, (*) indicates significant differences between basal and H_2O_2 treatment ($p < 0.05$).

Manuscript XII

Diet supplementation with vitamins E and C increases lymphocyte antioxidant protection in women affected by variegate porphyria who present impaired antioxidant defenses.

Ferrer MD, Tauler P, Sureda A, Palacín C, Tur JA, Pons A.

Diet supplementation with vitamins E and C increases lymphocyte antioxidant protection in women affected by variegate porphyria who present impaired antioxidant defenses

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Abstract
Variegate porphyria (VP) is the result of decreased protoporphyrinogen oxidase (PPOX) activity, and results in the accumulation of porphyrins and porphyrin precursors. Our aim was to analyze the influence of VP on the antioxidant defenses and oxidative damage markers of lymphocytes from variegate porphyria patients, together with the effects of diet supplementation with vitamins E and C on these parameters. Twelve women affected by VP and twelve pair matched healthy control women participated in a double-blind cross-over study. Each participant took either 50 mg/day vitamin E and 150 mg/day vitamin C or a placebo for six months. Neutrophil and monocyte counts were higher in the porphyric group. Catalase and glutathione peroxidase activities were reduced in variegate porphyria women. Catalase mRNA levels were not different between the two groups, while glutathione peroxidase expression was lower in the porphyric group. No significant differences were observed in the levels of oxidative damage markers such as malondialdehyde (MDA) and protein carbonyl derivatives. H_2O_2 production was higher in porphyric women after lymphocyte stimulation with PMA. Diet supplementation with antioxidant vitamins induced lymphopenia, but no effects were observed with respect to MDA, protein carbonyl derivative levels or H_2O_2 production. However, glutathione reductase and superoxide dismutase activities were higher in the enriched groups. In conclusion, women affected by variegate porphyria present a chronic inflammation-like condition and lymphocytes from these patients present a greater susceptibility to producing H_2O_2 and impaired H_2O_2 detoxifying mechanisms. Diet supplementation with vitamins E and C enhances the antioxidant activities of glutathione peroxidase and superoxide dismutase enzymes, although lymphocytes from variegate porphyria patients are still more susceptible to producing ROS.

Introduction

The porphyrias are a group of metabolic disorders of heme biosynthesis that result in the accumulation of porphyrins or their precursors [1, 2]. Although porphyrias are inherited diseases, there are some agents that can precipitate acute attacks and the appearance of clinical symptoms [3]. Variegate porphyria (VP), an autosomal dominant type of hepatic porphyria, is the result of decreased protoporphyrinogen oxidase (PPOX) activity, the penultimate enzyme of heme biosynthesis. It is characterized clinically by skin lesions and acute attacks that can occur separately or together [2]. The chronic accumulation of heme precursors in erythrocytes, liver and other cell types may induce cellular damage due to their ability to produce free radicals and to activate oxygen, inducing oxidative stress. Lymphocytes have been shown to be a good model to study ROS production and oxidative stress. When lymphocytes are stimulated with phorbol myristate acetate (PMA), ROS production increases [4, 5] by an unknown mechanism in which the PKC signaling pathway seems to be activated [6-8]. Antioxidant defenses in lymphocytes can be altered by oxidative stress in physiological situations of increased production of radical oxygen species (ROS) such as intense physical activity [9, 10], hypoxia/reoxygenation [11, 12] and scuba diving [4].

Antioxidant defenses and oxidative stress have been studied in some types of porphyria, but not in VP. Decreased plasma antioxidant vitamins levels and increased oxidative damage markers have been described in porphyria cutanea tarda patients [13, 14]. In contrast, no differences have been found in the levels of antioxidant vitamins or oxidative damage markers in acute intermittent porphyria patients [15]. The lower PPOX activity in the lymphocytes of variegate porphyria patients could

compromise heme biosynthesis and the level and function of heme proteins, as occurs in other types of porphyria.

Nutritional interventions with vitamin E and other antioxidants such as vitamin C or melatonin have been described to decrease the urinary levels of porphyrins or their precursors in patients affected by different types of porphyria [16-20]. However, other treatments with antioxidants have failed to obtain beneficial results [21, 22]. The failure to note a clinical response to antioxidant therapy may be due to factors dependent upon dosage of, or interaction between, the antioxidant compounds given, or on restricted bioavailability of the antioxidants at critical anatomical sites, and does not per se invalidate the model of acute porphyria as a hyperoxidative condition [22]. In any case, diet supplementation with antioxidant nutrients has been shown to decrease the levels of oxidative damage markers in lipids, proteins and DNA and to activate antioxidant enzymes after physical activity [23-27]. We have previously described that the prolonged consumption of high doses of vitamins C and E brings about an increase in the activity of antioxidant enzymes such as superoxide dismutase and catalase in neutrophils of healthy sportsmen [27]. The effect of this kind of nutritional intervention in the antioxidant defenses and oxidative damage markers of variegate porphyria patients is as yet unknown.

Therefore, we hypothesized that lymphocytes from variegate porphyria patients would show higher levels of mitochondrial ROS production and oxidative damage markers and impaired mitochondrial antioxidant defenses when compared to healthy women. Diet supplementation with antioxidant nutrients such as vitamins E and C should lead to an amelioration of the antioxidant status of women affected by variegate porphyria. Our aim was to analyze the influence of VP on the antioxidant defenses and oxidative damage markers of lymphocytes in variegate porphyria patients, and the effects of diet supplementation with vitamins E and C on these parameters.

Materials and methods

Subjects and study design

The study was performed with twelve women affected by VP and twelve pair matched healthy control women. All the patients participating in our study had been previously diagnosed as porphyric on the basis of different parameters such as plasma fluorescence peak at 626 nm, levels of excreted urinary and fecal porphyrins and clinical manifestations such as abdominal pain during porphyric attacks. All the subjects were informed of the purpose and demands of the study before giving their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the CAIB (Palma de Mallorca, Balearic Islands, Spain). The subjects participated in a double-blind cross-over study. Each participant drank, for six months, 500 ml/day almond-based beverage enriched with vitamin E (10 mg/100 ml) and vitamin C (30 mg/100 ml) or an identical beverage not enriched with antioxidant vitamins. Three months after finishing the first supplementation period the groups were crossed-over and another supplementation was performed for six months. Therefore, each subject was supplemented with both placebo and supplemented beverages. Blood samples were obtained before the beginning and at the end of each supplementation period.

Venous blood samples were obtained from the antecubital vein of control and porphyric women in resting conditions after overnight fasting. Lymphocyte fraction was purified from whole blood following an adaptation of the method described by Boyum [4, 28].

Enzymatic determinations

All activities were determined in lymphocytes and neutrophils with a Shimadzu UV-2100 spectrophotometer at 37ºC immediately after sample collection and cell purification. Catalase (CAT) activity was measured by the spectrophotometric method of Aebi based on the decomposition of H_2O_2 [29]. Glutathione peroxidase (GPx) activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler [30] using H_2O_2 as substrate. Glutathione reductase (GR) activity was measured by a modification of the Goldberg and Spooner spectrophotometric method [31]. Superoxide dismutase (SOD) activity was measured by an adaptation of the method of McCord and Fridovich [32]. The xanthine/xanthine oxidase system was used to generate the superoxide anion in order to produce the reduction of cytochrome *c* which was monitored at 550 nm. The superoxide dismutase in the sample removed the superoxide anion and produced an inhibition of the cytochrome *c* reduction.

Vitamin determinations

Vitamin E was determined in lymphocytes and neutrophils. The extraction of liposoluble vitamins was carried out using n-hexane after deproteinization with ethanol containing 0.2% BHT. Vitamin E was determined by HPLC in the n-hexane extract after drying under a nitrogen current and redissolving in ethanol. The mobile phase consisted of 550:370:80 acetonitrile:tetrahydrofuran:H_2O. The HPLC was a Shimadzu with a diode array detector and the column was a Nova Pak, C_{18}, 3.9x150 mm. α-tocopherol was determined at 290 nm.

Ascorbate was determined by an HPLC method with electrochemical detection [33] after deproteinization with meta-phosphoric acid. The mobile phase consisted of 0.05 M sodium phosphate, 0.05 M sodium acetate, 189 μM dodecyltrimethylammonium chloride and 36.6 μM tetraoctylammonium bromide in 25:75 methanol: H_2O, pH 4.8. The HPLC system was a Shimadzu with a Waters Inc electrochemical detector and a Nova Pak, C_{18}, 3.9x300 mm column. The potential of the chromatographic detector was set at 0.7 V versus an Ag/AgCl reference electrode.

mRNA gene expression

mRNA expressions were determined by real time RT-PCR with 18S ribosomal as the reference gene. For this purpose, mRNA was isolated from lymphocytes by phenol-chloroform extraction. cDNA was synthesized from 1 μg total RNA using reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. The primers used are shown in Table 1. For all PCRs there was one cycle at 95ºC for 10 min, followed by 40 cycles under the conditions shown in Table 1.

The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Basal mRNA levels at the beginning of the stage were arbitrarily referred to as 1. The expression of the target gene was normalized with respect to ribosomal 18S.

Malondialdehyde determination

Malondialdehyde (MDA) as a marker of lipid peroxidation was analyzed in lymphocytes and neutrophils using a colorimetric assay kit (Calbiochem, San Diego, CA, USA). The method used is specific for malondialdehyde determination.

Protein carbonyl derivate determination

Protein carbonyl derivates were measured in lymphocytes and neutrophils by an adaptation of the method of Levine [34] using the precipitates of deproteinized samples. Precipitates were re-suspended with 2,4-dinitrophenylhydrazine (DNPH) 10 mM, and incubated for 60 min at 37ºC. Then samples were precipitated with 20% trichloroacetic acid, and centrifuged for 10 min at 1000*g* at 4ºC. The precipitate was washed twice with ethanol:ethyl acetate (1:1) to remove free DNPH. 6 M Guanidine in 2 mM phosphate buffer, pH 2.3, was added to the precipitate, and samples were incubated for 40 min at 37ºC. Finally, samples were centrifuged for 5 min

Table 1. Primers and conditions used in Real Time PCRs

Gene	Primers	Conditions
18S	Fw: 5'-ATG TGA AGT CAC TGT GCC AG-3' Rv: 5'-GTG TAA TCC GTC TCC ACA GA-3'	95ºC,10 s; 60ºC, 7 s; 72ºC, 12 s
Catalase	Fw: 5'-TTT GGC TAC TTT GAG GTC AC-3' Rv: 5'-TCC CCA TTT GCA TTA ACC AG-3'	95ºC, 10 s; 60ºC, 10 s; 72ºC, 15 s
GPx	Fw: 5'-TTC CCG TGC AAC CAG TTT G-3' Rv: 5'-TTC ACC TCG CAC TTC TCG AA-3'	94ºC, 1 s; 60ºC, 7 s; 72ºC, 5 s
HO-1	Fw: 5'-CCA GCG GGC CAG CAA CAA AGT GC-3' Rv: 5'-AAG CCT TCA GTG CCC ACG GTA AGG-3'	95ºC, 0 s; 60ºC, 5 s; 72ºC, 10s
GRd	Fw: 5'-TCA CGC AGT TAC CAA AAG GAA A-3' Rv: 5'-CAC ACC CAA GTC CCC TGC ATA T-3'	95ºC, 30 s; 64ºC, 45 s; 72ºC, 30 s
Mn-SOD	Fw: 5'-GAG AAG TAC CAG GAG GCG TTG-3' Rv: 5'- CAA GCC AAC CCC AAC CTG AGC-3'	95ºC, 30 s; 64ºC, 60 s
Cu/Zn-SOD	Fw: 5'-TCA GGA GAC CAT TGC ATC ATT-3' Rv: 5'-CGC TTT CCT GTC TTT GTA CTT TCT TC-3'	95ºC, 30 s; 64ºC, 45 s; 72ºC, 30 s

at 3000*g* at 4ºC to clarify the supernatant and absorbance was measured at 360 nm. The molar absorption of 22,000 $M^{-1} \cdot cm^{-1}$ was used to quantify the protein carbonyl levels. Samples were analyzed against a blank of guanidine solution.

Hydrogen peroxide production

H_2O_2 production in lymphocytes was measured before and after stimulation with phorbol myristate acetate (PMA) using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as an indicator. DCFH-DA (30 μg/ml) in PBS was added to a 96-well microplate containing 50 μl lymphocyte suspension. PMA (3 μM) prepared in HBSS or HBSS alone was added to the wells and the fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37ºC for 1 hour in a FLx800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc.).

Statistical analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 13.0 for Windows). Results are expressed as mean ± s.e.m. and $P<0.05$ was considered statistically significant. The statistical significance of the data was assessed by Student's t-test for unpaired data.

Results

The leukocyte formula of porphyric and control patients at the beginning of the study is shown in Table 2. The neutrophil count, as well as monocyte count, was significantly higher in the porphyric group when compared to the control group. Lymphocyte, eosinophil and basophil counts were not different between the two groups.

Variegate porphyria affected the activity of lymphocyte antioxidant enzymes before supplementation (Table 3). The activities of the H_2O_2 detoxifying enzymes, catalase and glutathione peroxidase, were reduced in variegate porphyria patients when compared to controls (80% and 75%, respectively), while glutathione reductase and superoxide dismutase showed similar activities in both groups. Gene expression of both catalase and glutathione peroxidase was then measured to better understand the increased activities of such enzymes (Figure 1). Catalase mRNA levels were not different between the two groups. However, glutathione peroxidase mRNA levels were about 75% in the porphyric group when compared to the control group, as happened with the enzyme activity. Hemeoxygenase 1 expression, measured as a marker of oxidative stress, did not show significant differences between the porphyric and control groups.

Table 2. Effect of variegate porphyria on leukocyte formula.

	Control	Porphyria
Leukocytes (10^3/μl)	6.04 ± 0.23	6.66 ± 0.22
Neutrophils (10^3/μl)	3.31 ± 0.16	3.87 ± 0.16 #
Lymphocytes (10^3/μl)	2.06 ± 0.10	1.99 ± 0.09
Monocytes (10^3/μl)	0.323 ± 0.012	0.421 ± 0.025 #
Eosinophils (10^3/μl)	0.240 ± 0.037	0.229 ± 0.025
Basophils (10^3/μl)	0.0290 ± 0.0032	0.0294 ± 0.0016

Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups ($p < 0.05$).

No significant differences were observed in the levels of the antioxidant vitamins ascorbate and α-tocopherol or in the levels of oxidative damage markers such as malondialdehyde (MDA) and protein carbonyl derivatives at the beginning of the diet intervention (Table 3). In relation to these results, H_2O_2 production in lymphocytes in basal conditions was similar in porphyric and control patients (Figure 2). After stimulation with PMA,

Table 3. Effect of variegate porphyria on lymphocyte antioxidants and oxidative damage markers.

	Control	Porphyria
Catalase (K/10^9 cell)	14.3 ± 1.3	11.3 ± 0.8 #
GPx (nkat/10^9 cell)	115 ± 9	86.8 ± 6.5 #
GRd (nkat/10^9 cell)	53.3 ± 3.2	51.2 ± 3.3
SOD (pkat/10^9 cell)	26.9 ± 2.2	24.0 ± 2.2
Ascorbate (mM)	10.4 ± 0.6	9.60 ± 0.59
α-tocopherol (μM)	537 ± 29	554 ± 32
MDA (μM/10^9 cell)	0.986 ± 0.267	0.748 ± 0.099
Carbonyl derivatives (μmol/10^9 cell)	46.2 ± 3.4	46.7 ± 5.0

Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups ($p < 0.05$).

Table 4. Effects of a diet supplementation with vitamins E and C on leukocyte formula of variegate porphyria patients.

		Control	Porphyria	ANOVA D	ANOVA S	ANOVA D*S
Leukocytes	**Non-enriched**	7.13 ± 0.58	6.57 ± 0.44			
(10^3/μl)	**Enriched**	5.96 ± 0.47	6.19 ± 0.37			
Neutrophils	**Non-enriched**	4.09 ± 0.58	3.75 ± 0.29			
(10^3/μl)	**Enriched**	3.35 ± 0.36	3.53 ± 0.19			
Lymphocytes	**Non-enriched**	2.25 ± 0.13	2.07 ± 0.12			
(10^3/μl)	**Enriched**	1.89 ± 0.13	1.86 ± 0.16		x	
Monocytes	**Non-enriched**	0.299 ± 0.026	0.374 ± 0.040			
(10^3/μl)	**Enriched**	0.316 ± 0.022	0.403 ± 0.068	x		
Eosinophils	**Non-enriched**	0.316 ± 0.083	0.218 ± 0.055			
(10^3/μl)	**Enriched**	0.265 ± 0.064	0.228 ± 0.0614			
Basophils	**Non-enriched**	0.0491 ± 0.0060	0.0433 ± 0.0053			
(10^3/μl)	**Enriched**	0.0379 ± 0.0063	0.0425 ± 0.0045			

Statistical analysis: Two-way ANOVA. (D) significant effect of disease; (S) significant effect of supplementation. (DxS) significant interaction between the two ANOVA factors ($p < 0.05$).

however, lymphocytes from porphyric women produced greater amounts of H_2O_2 than control women.

Diet supplementation with either an almond based beverage or an almond based beverage enriched with vitamins C and E was carried out for six months and after this period the same determinations were performed. The effects of supplementation on the leukocyte formula are shown in Table 4. After the supplementation period the differences in neutrophil counts between porphyric and control groups were not evident in either supplementation group. With respect to the monocyte count, however, the previously described effect of disease was maintained after the supplementation period, with porphyric patients showing higher monocyte counts than controls. An effect of type of supplementation was found in the lymphocyte counts. Both porphyric and control subjects supplemented with the antioxidant enriched beverage showed lower lymphocyte counts than subjects supplemented with the non-enriched drink.

The type of beverage consumed affected the lymphocyte levels of α-tocopherol (Table 5). In both control and porphyric groups, subjects consuming the enriched drink presented α-tocopherol levels 150% higher than subjects consuming the non-enriched drink, although the difference was only significant in the control subjects. Ascorbate levels were not affected by the type of beverage consumed but by disease. The porphyric and control groups consuming the non-enriched drink presented similar values of ascorbate levels in lymphocytes. However, significant differences were found between porphyric and control patients who consumed the enriched drink, with porphyric women showing lower ascorbate levels than controls. No effects of disease or type of supplementation were observed with respect to MDA and protein carbonyl derivatives levels (Table 5) after the intervention. However, H_2O_2 production (Figure 3) was affected by the porphyric condition as happened prior to the intervention, with lymphocytes from porphyric women producing higher amounts of H_2O_2 after stimulation with PMA. The supplementation with antioxidant vitamins did not affect H_2O_2 production by lymphocytes.

The effects of supplementation on antioxidant enzyme activities are shown in Table 6. Catalase and glutathione peroxidase activities were not affected by porphyria or type of supplementation. Glutathione reductase and superoxide dismutase activities, however, were affected by the type of supplementation. Both antioxidant enzymes showed greater activities in the enriched groups when compared to the non-enriched, in both control and porphyric women. An effect of disease was also observed in superoxide dismutase activity, with higher activities in the porphyric groups. The gene expression of all these antioxidant enzymes was then measured. After the six months of supplementation with the almond based beverage no effects of variegate porphyria were observed in the expression levels of glutathione reductase, glutathione peroxidase, Mn-superoxide dismutase or Cu/Zn-superoxide dismutase (Figure 4). Neither did the enrichment of the beverage with vitamins C and E affect the expression of antioxidant enzymes in lymphocytes.

Table 5. Effects of a diet supplementation with vitamins E and C on antioxidant vitamins and oxidative damage markers in variegate porphyria patients.

		Control	Porphyria	ANOVA D	S	D*S
Ascorbate (mM)	**Non-enriched**	7.54 ± 0.95	6.50 ± 0.80			
	Enriched	9.58 ± 1.22	6.52 ± 1.00 #	x		
α-tocopherol (μM)	**Non-enriched**	551 ± 50	511 ± 70			
	Enriched	797 ± 57 §	750 ± 134		x	
MDA ($\mu M/10^9$ cell)	**Non-enriched**	1.24 ± 0.25	0.967 ± 0.233			
	Enriched	1.10 ± 0.21	1.34 ± 0.25			
Carbonyl derivatives ($\mu mol/10^9$ cell)	**Non-enriched**	30.3 ± 2.6	36.8 ± 5.6			
	Enriched	38.7 ± 4.7	44.9 ± 11.3			

Statistical analysis: Two-way ANOVA. (D) significant effect of disease; (S) significant effect of supplementation. (DxS) significant interaction between the two ANOVA factors. (#) indicates significant differences between porphyria and control groups; (§) indicates significant differences between non-enriched and enriched beverage groups ($p < 0.05$).

Discussion

Variegate porphyria is the result of decreased PPOX activity, the penultimate enzyme of heme biosynthesis. This disease is characterized by an accumulation of heme precursors and by a low rate of heme biosynthesis as a result of the genetic error in PPOX gene. All the patients participating in this study had been previously diagnosed as porphyric on the basis of different parameters such as plasma fluorescence peak at 626 nm, levels of excreted urinary and fecal porphyrins, and clinical manifestations. The leukocyte formula was altered in the women affected by porphyria. Total leukocyte count was slightly higher in the porphyric group, although the difference was not significant. This was due to significantly higher neutrophil and monocyte counts. Monocytes are cells that respond to inflammation signals by moving to the site of infection in tissues and differentiating into macrophages to perform an immune response. Neutrophils are phagocytic leukocytes normally found in the blood stream, but which can migrate to tissues following inflammation signals. In this instance, in situations of inflammation, blood neutrophil counts are increased. The fact that both monocyte and neutrophil counts are higher in porphyric women than their controls could be indicative of a situation of greater inflammation in these patients. However, it must be noted that both neutrophil and monocyte levels in porphyrics are within the normal range.

The malfunction of PPOX in lymphocytes could compromise the heme synthetic pathway and hence alter the level and function of heme proteins. We found that catalase activity was decreased in porphyric women when compared to controls, which could be the result of limitations in free heme

Table 6. Effects of a diet supplementation with vitamins E and C on antioxidant enzyme activities in variegate porphyria patients.

		Control	Porphyria	ANOVA D	S	D*S
Catalase ($K/10^9$ cell)	**Non-enriched**	14.5 ± 1.3	14.2 ± 1.1			
	Enriched	16.6 ± 2.0	13.1 ± 0.8			
GPx ($nkat/10^9$ cell)	**Non-enriched**	140 ± 19	166 ± 17			
	Enriched	153 ± 13	133 ± 8			
GRd ($nkat/10^9$ cell)	**Non-enriched**	40.1 ± 2.0	36.0 ± 8.6			
	Enriched	58.4 ± 3.3 §	52.5 ± 6.6 §		x	
SOD ($pkat/10^9$ cell)	**Non-enriched**	41.4 ± 3.9	54.0 ± 4.3			
	Enriched	60.8 ± 5.1 §	71.7 ± 7.8 §	x	x	

Statistical analysis: Two-way ANOVA. (D) significant effect of disease; (S) significant effect of supplementation. (DxS) significant interaction between the two ANOVA factors. (#) indicates significant differences between porphyria and control groups; (§) indicates significant differences between non-enriched and enriched beverage groups ($p < 0.05$).

biosynthesis since catalase is a heme protein which requires the heme group to catalyze H_2O_2 decomposition. In a similar way, glutathione peroxidase activity was also decreased. Like catalase, glutathione peroxidase also detoxifies H_2O_2, but through a different mechanism. For this reason we studied the gene expression of both catalase and glutathione peroxidase. Catalase expression was similar in the porphyric and control groups, reinforcing the possibility that the impaired catalase activity is due to limitations in the heme group. Once synthesized the catalase subunit protein itself seems to fold without heme into a conformation similar to the holoenzyme subunit. Heme binding may act to optimize subunit conformation for contact with another subunit [35]. Thus, decreased heme availability could lead to incomplete catalase assembly and therefore result in decreased catalase activity. Glutathione peroxidase expression, however, was decreased in the porphyric women to a similar extent as enzyme activity, indicating that the lower enzyme activity is due to a down-regulation of the gene expression rather than a direct modification in the activity.

The accumulation of heme precursors has been shown to constitute an endogenous source of reactive oxygen species, triggering oxidative damage to cellular components [36, 37]. Therefore, H_2O_2 production in lymphocytes as an indicator of intracellular ROS formation was studied. Lymphocytes from porphyric and control women produced approximately the same amount of H_2O_2 in basal conditions. However, greater H_2O_2 production was detected in the porphyric group compared to controls when lymphocytes were activated with PMA. These results show that lymphocytes from porphyric women are more susceptible to producing ROS and could then be more vulnerable to suffering from oxidative damage. The mechanisms by which ROS production is increased in lymphocytes after PMA activation is not clearly understood as yet but the activation of PKC signaling pathway seems to be involved [6-8]. It has also been evidenced that lymphocytes from porphyric women present impaired capability to detoxify H_2O_2. The combination of both impaired antioxidant defenses and increased ROS production could lead to a situation of oxidative stress, which is why we measured MDA and protein carbonyl levels as indicators of oxidative damage. No evidence of lipid or protein oxidation was found in the porphyric patients in basal conditions. Our results confirm that in basal conditions, lymphocytes from women affected by variegate porphyria present impaired antioxidant defenses, but this does not result in a situation of oxidative stress. However, when reactive oxygen species formation is increased, the lymphocyte antioxidant capacity is overwhelmed and oxidative damage may occur.

Diet supplementation with antioxidants could be appropriate in variegate porphyric patients in view of these results. Diet supplementation with vitamin E has been shown to be effective in reducing porphyrin levels in patients affected by porphyria cutanea tarda [38-40]. However, few studies have been performed concerning the effects of an antioxidant supplementation on variegate porphyria patients, and these studies have been focused on the effects on symptoms or porphyrin excretion rather than on the oxidative stress condition of patients [41, 42]. In our double-blind cross-over study, porphyric and healthy women were supplemented with vitamins C and E (150mg/d and 50mg/d, respectively) for six months, using an almond based beverage as the carrier and placebo. The supplementation with antioxidant vitamins was effective in increasing the lymphocyte levels of α-tocopherol. Ascorbate levels in lymphocytes also tended to increase in the supplemented group, but this tendency was only observed in the control patients. These results show that lymphocytes from porphyric women are more resistant to accumulating intracellular ascorbate, maybe suggesting a higher utilization of this vitamin to counteract oxidative stress. The supplementation with antioxidant vitamins induced a decrease in the number of circulating lymphocytes, but the lymphocyte count stayed within the normal range. Although some studies have revealed that supplementation with vitamin E can increase lymphocyte proliferation [43], in a previous experiment in which amateur runners consumed the same supplement we also detected a decrease in the number of circulating lymphocytes [44]. Supplementation with either 1 g/d vitamin C and 500 mg/d vitamin E [45] or 1 g/d of vitamin C alone [46] has also been shown to induce lymphopenia. The mechanisms by which the supplementation with antioxidant vitamins induces lymphopenia are not clear, although an increase in the levels of corticosteroids in response to ascorbate could be involved [47, 48].

The differences in catalase and glutathione peroxidase activities between porphyric and control patients observed in initial conditions were not evidenced after the supplementation period. Diet supplementation with vitamin C and vitamin E induced greater glutathione reductase and superoxide dismutase activities. These changes in enzyme activity were not accompanied by changes in the gene expression, thus suggesting that the

regulation carried out by the supplementation with antioxidant vitamins is at a post-transcriptional level. There is evidence of a direct activation of some enzymes such as glutathione reductase and superoxide dismutase, as a result of post-transcriptional events [49, 50]. We previously evidenced in an *in vitro* experiment that glutathione reductase activity increases when measured in the presence of catalase [49]. Furthermore, we have previously observed increases in glutathione reductase activity and superoxide dismutase in erythrocytes after a cycling stage [50]. As erythrocytes can not express genes or synthesize new proteins, these previous results indicate that changes in glutathione reductase and superoxide dismutase activities can be attributed to direct effects on the protein. These effects increase the catalytic capabilities of these enzymes. Glutathione reductase is a flavoprotein which could transfer one electron to oxygen in the course of the redox reaction leading to ROS formation as occurs in other flavoproteins such as xanthine oxidase [51] and nitric oxide synthetase [52]. The ROS produced by the enzyme could affect the integrity of the protein and impair its own activity; the presence of higher antioxidant levels such as increased tocopherol or ascorbate in lymphocytes could contribute to the scavenging of ROS and then maintain higher glutathione reductase activities. A physiological role of increased glutathione reductase activities as a result of antioxidant supplementation could lie in the recycling mechanisms of both tocopherol and ascorbate. After acting as antioxidants, both vitamins get oxidized. The reduced forms can be recycled by glutathione or enzymatically in reactions using glutathione [53, 54]. In these cases, glutathione is oxidized and glutathione reductase activity is needed to recover reduced glutathione. An increase in vitamin C and vitamin E levels could lead to greater ascorbate and tocopherol utilization and recycling, hence increased glutathione reductase activities would be needed in order to recover reduced glutathione. Either way, we can suspect an effect of the increased intracellular levels of vitamin E in lymphocytes in the activities of some antioxidant enzymes such as glutathione reductase and superoxide dismutase as occurs in the erythrocyte [50]. After the supplementation period, lymphocytes from variegate porphyria women were still more susceptible to producing ROS when activated with PMA, but the increased antioxidant enzyme activities in the group supplemented with vitamins C and E provided a higher degree of defenses against the induction of oxidative stress.

The main findings of our study are that women affected by variegate porphyria present a chronic inflammation-like condition with higher neutrophil and monocyte circulating counts and lymphocytes from these patients presenting a greater susceptibility to producing H_2O_2 and impaired H_2O_2 detoxifying mechanisms. Diet supplementation with vitamin E (50 mg/day) and vitamin C (150 mg/day) turns out to be effective in increasing the lymphocyte levels of α-tocopherol and enhances the antioxidant activities of glutathione peroxidase and superoxide dismutase enzymes, although lymphocytes from variegate porphyria patients are still more susceptible to producing ROS.

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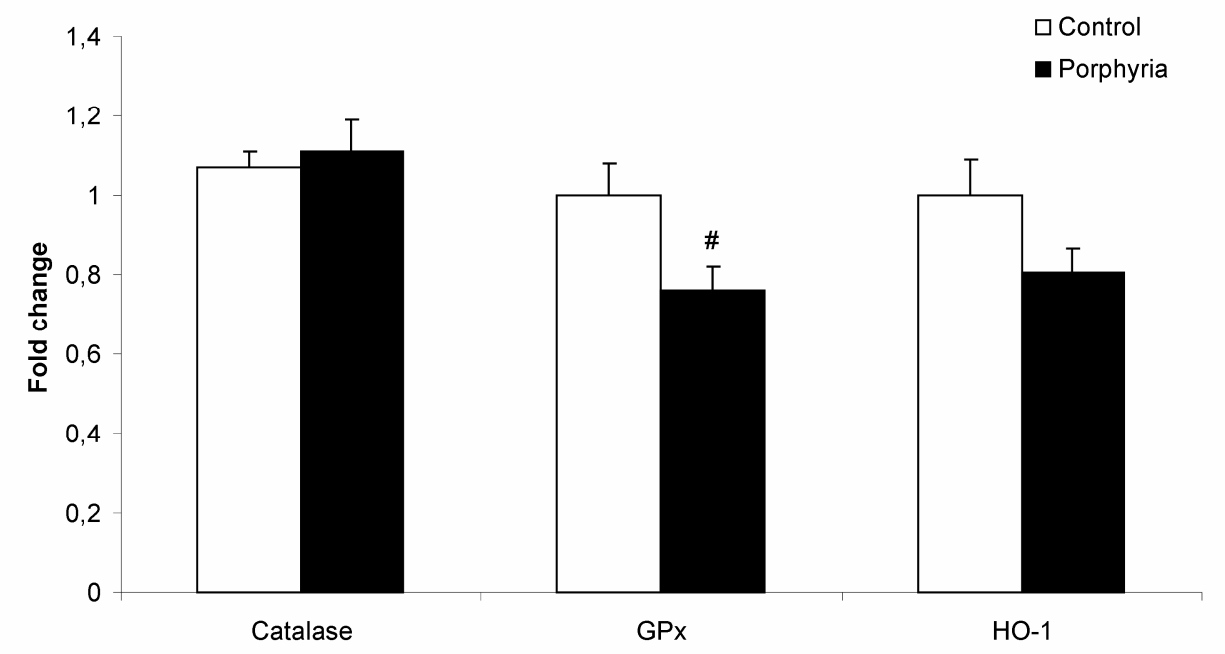
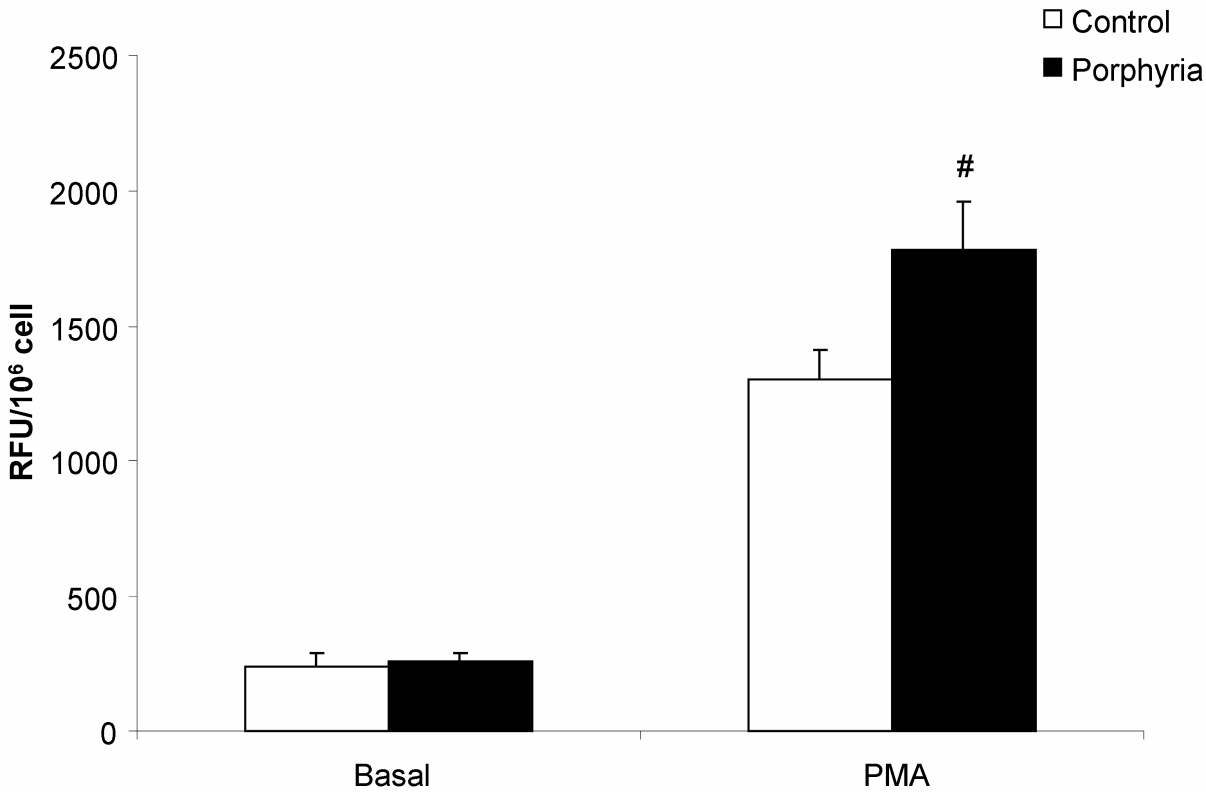
FIGURES

Figure 1. Effects of variegate porphyria on lymphocyte catalase, glutathione peroxidase (GPx) and hemeoxygenase-1 (HO-1) gene expressions.



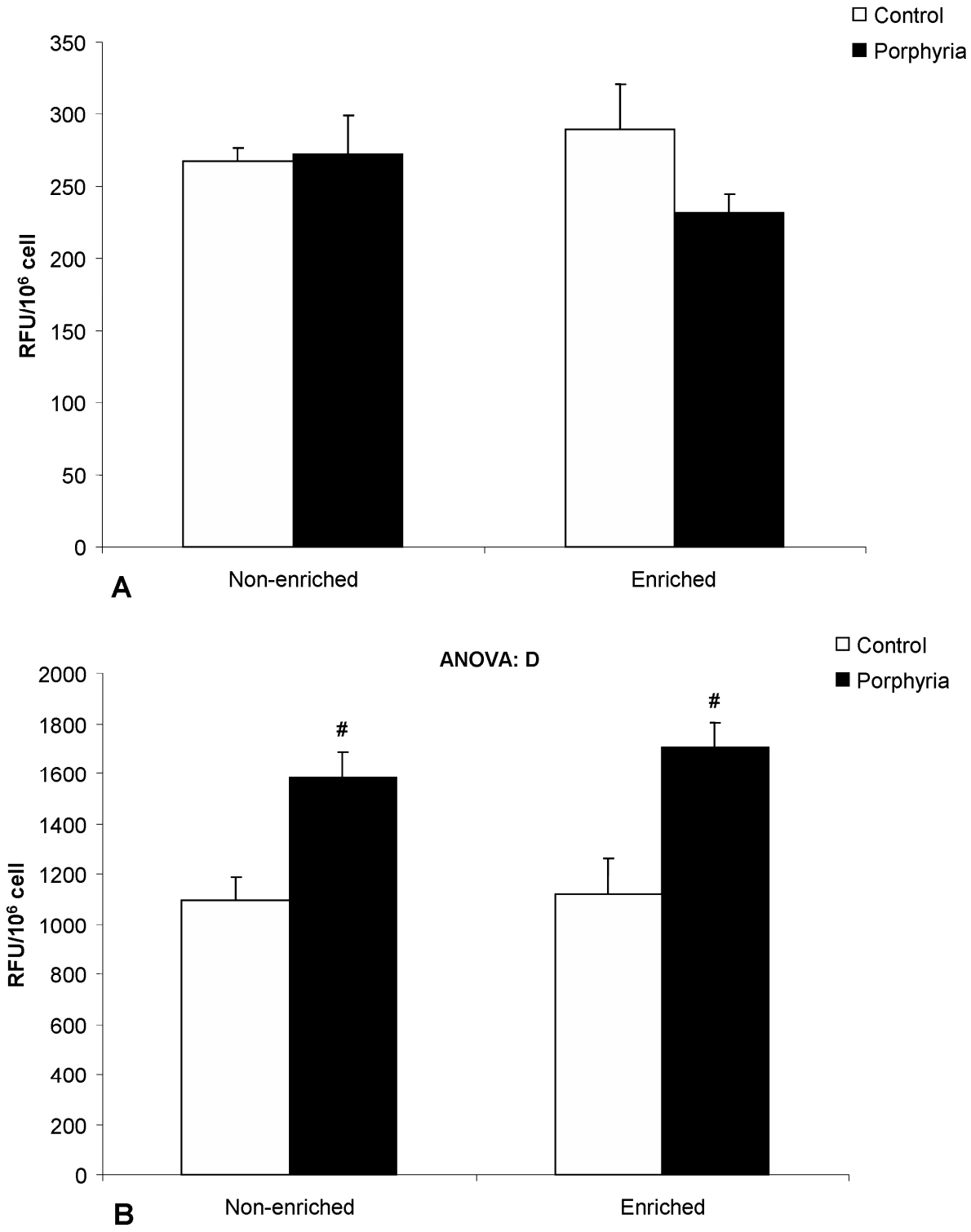
The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups ($p < 0.05$).

Figure 2. Effects of variegate porphyria on lymphocyte H_2O_2 production.



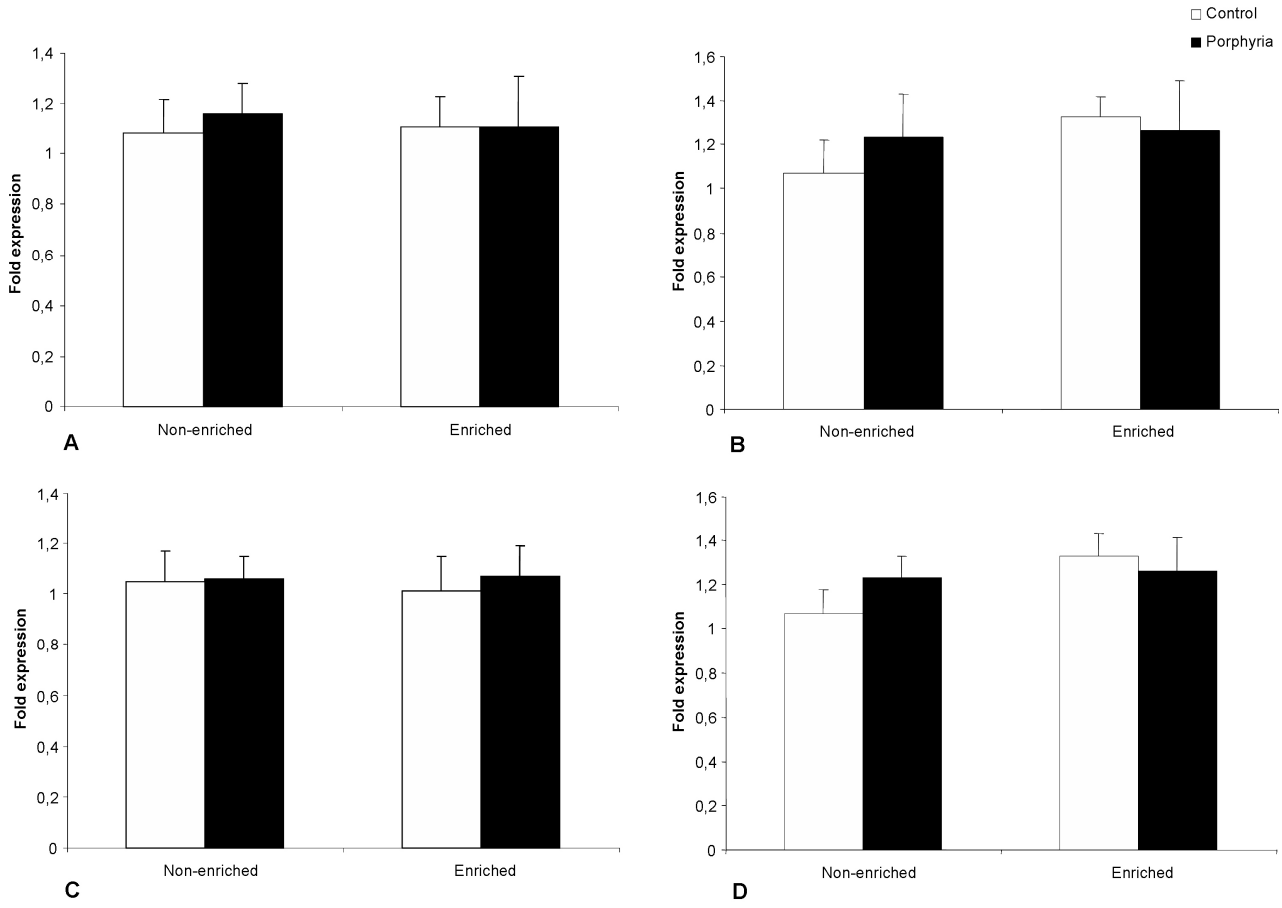
Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups ($p < 0.05$).

Figure 3. Effects of diet supplementation with vitamins E and C on lymphocyte H_2O_2 production in basal conditions (A) and after lymphocyte stimulation with PMA (B).



Statistical analysis: Two-way ANOVA. (D) significant effect of disease. (#) indicates significant differences between porphyria and control groups ($p < 0.05$).

Figure 4. Effects of diet supplementation with vitamins E and C on lymphocyte glutathione peroxidase (A), glutathione reductase (B), Mn-SOD (C) and Cu/Zn-SOD (D) gene expression.



The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. Statistical analysis: Two-way ANOVA. No significant effects were evidenced ($p < 0.05$).

Manuscript XIII

Variegate porphyria induces plasma and neutrophil oxidative stress. Effects of a diet supplementation with vitamins E and C.

Ferrer MD, Tauler P, Sureda A, Palacín C, Tur JA, Pons A.

Variegate porphyria induces plasma and neutrophil oxidative stress. Effects of a diet supplementation with vitamins E and C.

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Abstract
Variegate porphyria (VP) is the result of decreased protoporphyrinogen oxidase (PPOX) activity. Our aim was to analyze the influence of VP in the antioxidant defenses and markers of oxidative damage and inflammation in plasma and neutrophils and the effects of a diet supplementation with vitamins E and C on these parameters in plasma, neutrophils and erythrocytes. Twelve women affected by VP and twelve paired matched control healthy women participated in a double-blinded cross-over study. Each participant intook 50 mg/day of vitamin E and 150 mg/day of vitamin C, or a placebo, during six months, by consuming an almond based beverage as the vehicle. Women affected by VP presented higher c-reactive protein and malondialdehyde (MDA) circulating levels. Plasma antioxidant defenses (ascorbate, α-tocopherol, uric acid, bilirubin and transferrin) were not different between porphyric and control women. Neutrophils from VP women presented decreased catalase and glutathione peroxidase activities, together with increased protein carbonyl levels. Reactive oxygen species production from stimulated neutrophils was also higher in porphyric women than their controls. The diet supplementation was effective in increasing α-tocopherol levels in neutrophils and in reducing MDA levels in plasma. Neutrophil antioxidant enzyme activities were unaffected by the diet supplementation. Erythrocyte catalase and glutathione reductase activities were enhanced by the enriched beverage, only in the control subjects. In conclusion, women affected by variegate porphyria present a situation of inflammation, plasma oxidative damage and more primed neutrophils to oxidative burst, with decreased antioxidant activities and increased ROS production capabilities and protein oxidative damage. The diet supplementation with vitamin E (50 mg/day) and vitamin C (150 mg/day) during six months decreases plasma oxidative damage and enhances the erythrocyte activities of catalase and glutathione reductase.

Introduction

The porphyrias are a group of metabolic disorders of heme biosynthesis that result in the accumulation of porphyrins or their precursors [1, 2]. Although people affected by porphyria can remain asymptomatic for a long period of time or even for a lifetime, there are some agents that can precipitate acute attacks and the appearance of clinical symptoms [3]. Variegate porphyria (VP), an autosomal dominant type of hepatic porphyria, is the result of decreased protoporphyrinogen oxidase (PPOX) activity, the penultimate enzyme of heme biosynthesis. It is characterized clinically by skin lesions and acute attacks that can occur separately or together [2]. The chronic accumulation of heme precursors in erythrocytes, liver and other cell types can induce cellular damage due to their ability to produce free radicals and to activate oxygen, inducing oxidative stress.

Antioxidant defenses and oxidative stress have been studied in some types of porphyria, but not in VP. Decreased plasma antioxidant vitamins levels and increased oxidative damage markers were described in porphyria cutanea tarda patients [4, 5]. In contrast, no differences have been found in the levels of antioxidant vitamins or oxidative damage markers in acute intermittent porphyria patients [6]. Therefore it is necessary to describe the levels of antioxidant defenses and oxidative damage markers in variegate porphyria patients in order to characterize this porphyria as a prooxidative disease. The determination of these parameters in plasma is a good marker of the systemic situation, but studying concrete cell types should be useful to further understand the impact of the disease in the normal function of cells. Neutrophils are phagocytic leukocytes normally found in the blood stream, but they can migrate to tissues following inflammation signals. In this instance, in situations of

inflammation blood neutrophil counts are increased. When neutrophils are activated in response to immune stimulation they get primed to the oxidative burst, in which large amounts of reactive oxygen species (ROS) are produced. The toxicity of ROS produced by neutrophils could damage the neutrophil itself and adjacent tissues contributing to the oxidative stress situation [7, 8]. In order to face this high production of ROS, neutrophils contain antioxidant enzymes such as superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPx). These antioxidant enzymes are known to be regulated by inflammatory cytokines, oxygen tension and ROS in human neutrophils [9]. We have previously described that exercise induces an acute phase immune response in neutrophils, by increasing their number in circulation, getting primed to oxidative burst and decreasing their antioxidant enzyme activities, possibly due to enzyme release to plasma [10]. In addition to the possible oxidative stress induced by the accumulation of heme precursors in the neutrophil, the minor PPOX activity in variegate porphyria patients could compromise per se the heme biosynthesis and the level and function of heme proteins.

It has been described that nutritional interventions with vitamin E and other antioxidants such as vitamin C or melatonin decrease the urinary levels of porphyrins or their precursors in patients affected by different types of porphyria [11-15]. However, other treatments with antioxidants have failed to obtain beneficial results [16, 17]. The failure to note a clinical response to antioxidant therapy may be due to factors dependent upon dosage of, or interaction between, the antioxidant compounds given, or on restricted bioavailability of the antioxidants at critical anatomical sites, and does not per se invalidate the model of acute porphyria as a hyperoxidative condition [17]. In any case, diet supplementation with antioxidant nutrients has been shown to decrease the levels of oxidative damage markers in lipids, proteins and DNA and to activate antioxidant enzymes after physical activity [18-22]. We have previously described that the prolonged consumption of high doses of vitamins C and E induce an increase in the activity of antioxidant enzymes such as superoxide dismutase and catalase in neutrophils of healthy sportsmen [22]. The effect of this kind of nutritional intervention in the antioxidant defenses and oxidative damage markers of variegate porphyria patients is unknown. Therefore, we hypothesized that women affected by variegate porphyria would present evidences of a systemic situation of oxidative stress and inflammation, and that neutrophils from variegate porphyria patients would be in an activated state. The diet supplementation with antioxidant nutrients such as vitamins E and C should lead to ameliorate the antioxidant status of women affected by variegate porphyria by decreasing oxidative damage and/or enhancing the endogenous antioxidant defenses. Our aim was to analyze the influence of VP in the antioxidant defenses and markers of oxidative damage and inflammation in plasma and neutrophils from variegate porphyria patients, and the effects of a diet supplementation with vitamins E and C on these parameters.

Materials and methods

Subjects and study design

The study was performed with twelve women affected by VP and twelve paired matched control healthy women. All the patients participating in our study had been previously diagnosed as porphyric on the basis of different parameters such as plasma fluorescence peak at 626 nm, levels of excreted urinary and faecal porphyrins and clinical manifestations such as abdominal pain during porphyric attacks. All the subjects were informed of the purpose and demands of the study before giving their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the CAIB (Palma de Mallorca, Balearic Islands, Spain). The subjects participated in a double-blinded cross-over study. Each participant drunk, for six months, 500 ml/day of an almond-based beverage enriched with vitamin E (10 mg/100 ml) and vitamin C (30 mg/100 ml) or an identical beverage but not enriched with antioxidant vitamins. Three months after finishing the first supplementation period the groups were crossed-over and another supplementation was performed during six months. Therefore, each subject was supplemented with both placebo and supplemented beverages. Blood samples were obtained before the beginning and at the end of every supplementation period.

Venous blood samples were obtained from the antecubital vein of control and porphyric women in resting conditions after overnight fasting. Plasma, neutrophil and erythrocyte fractions were purified from whole blood following an adaptation of the method described by Boyum [23, 24].

Serum clinical analysis

Gamma glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH), creatine kinase (CK), C reactive protein, uric acid, bilirrubin were determined by standard procedures using commercial clinical kits in an autoanalyser system (Technicon DAX System). Transferrin was measured by immuno-precipitation using the ILAB 600 (Clinical Chemistry System, IZASA).

Enzymatic determinations

All activities were determined in neutrophils and erythrocytes with a Shimadzu UV-2100 spectrophotometer at 37ºC. Catalase (CAT) activity was measured by the spectrophotometric method of Aebi based on the decomposition of H_2O_2 [25]. Glutathione peroxidase (GPx) activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler [26] using H_2O_2 as substrate. Glutathione reductase (GR) activity was measured by a modification of the Goldberg and Spooner spectrophotometric method [27]. Superoxide dismutase (SOD) activity was measured by an adaptation of the method of McCord and Fridovich [28].The xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion produced the reduction of cytochrome *c*, which was monitored at 550 nm. The superoxide dismutase in the sample removed the superoxide anion and produced an inhibition of the cytochrome *c* reduction.

Vitamin determinations

Vitamin E was determined in plasma and neutrophils. The extraction of liposoluble vitamins was carried out using n-hexane after deproteinization with ethanol containing 0.2% BHT. Vitamin E was determined by HPLC in the n-hexane extract after drying under a nitrogen current and redissolving in ethanol. The mobile phase consisted of 550:370:80 acetonitrile:tetrahydrofuran:H_2O. The HPLC was a Shimadzu with a diode array detector and the column was a Nova Pak, C_{18}, 3.9x150 mm. α-tocopherol was determined at 290 nm.

Ascorbate was determined in plasma and neutrophils by an HPLC method with electrochemical detection [29] after deproteinization with meta-phosphoric acid. The mobile phase consisted of 0.05 M sodium phosphate, 0.05 M sodium acetate, 189 µM dodecyltrimethylammonium chloride and 36.6 µM tetraoctylammonium bromide in 25:75 methanol: H_2O, pH 4.8. The HPLC system was a Shimadzu with a Waters Inc electrochemical detector and a Nova Pak, C_{18}, 3.9x300 mm column. The potential of the chromatographic detector was set at 0.7 V versus an Ag/AgCl reference electrode.

Malondialdehyde determination

Malondialdehyde (MDA) as a marker of lipid peroxidation was analyzed in plasma and neutrophils by a colorimetric assay kit (Calbiochem, San Diego, CA, USA). The method used is specific for malondialdehyde determination.

Protein carbonyl derivate determination

Protein carbonyl derivates were measured in plasma and neutrophils by an adaptation of the method of Levine [30] using the precipitates of deproteinized samples. Precipitates were re-suspended with 2,4-dinitrophenylhydrazine (DNPH) 10 mM, and incubated for 60 min at 37ºC. Then samples were precipitated with 20% trichloroacetic acid, and centrifuged for 10 min at 1000*g* at 4ºC. The precipitate was washed twice with ethanol:ethyl acetate (1:1) to remove free DNPH. 6 M Guanidine in 2 mM phosphate buffer, pH 2.3, was added to the precipitate, and samples were incubated for 40 min at 37ºC. Finally, samples were centrifuged for 5 min at 3000*g* at 4ºC to clarify the supernatant and absorbance was measured at 360 nm. The molar absorption of 22,000 $M^{-1} \cdot cm^{-1}$ was used to quantify the protein carbonyl levels. Samples were analyzed against a blank of guanidine solution.

Chemiluminescence assay

Reactive oxygen species production was measured in neutrophils. Opsonized zymosan (OZ) was used as neutrophil stimulant. Zymosan A (Sigma) was suspended in HBSS at a concentration of 1 mg/ml and incubated with 10% human serum at 37ºC for 30 min to opsonize the zymosan, followed by centrifugation at 750xg for 10 min at 4ºC. The precipitate was washed twice in HBSS and finally resuspended in HBSS at 1 mg/ml.

Chemiluminescence assay was performed by an adaptation of the method by Edwards [31]. Luminol is a lumigenic probe which can be oxidized by H_2O_2 and HOCl. Thus, in activated neutrophils, luminol chemiluminescence measures the combined activities of the NADPH oxidase plus myeloperoxidase. OZ suspension (100 µl) was added to a 96-well microplate containing 50 µl neutrophil suspension and 50 µl luminol solution (2 mM in PBS, pH 7.4). Chemiluminescence was measured at

37ºC for 90 min in FLx800 Microplate Fluorescence Reader (Bio-tek Instruments Inc., USA). Each sample was determined in duplicate.

Statistical analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 13.0 for Windows). Results are expressed as mean ± s.e.m. and $P<0.05$ was considered statistically significant. The statistical significance of the data was assessed by Student's t-test for unpaired data. To test the effects of supplementation and disease, a two-way analysis of variance (ANOVA) was performed. The statistical factors analyzed were (D) disease and (S) supplementation. When significant effects were found, a one-way ANOVA was used to determine the differences between the groups involved.

Results

The porphyria disease did not affect the circulating activities of the enzymes gamma glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH) and creatine kinase (CK), shown in Table 1. The levels of the inflammation marker c-reactive protein were however increased in women affected of variegate porphyria when compared to controls. Porphyric patients presented higher MDA levels, while carbonyl was maintained in porphyric patients. The higher levels of oxidative damage in plasma of variegate porphyria patients could be a consequence of decreased circulating antioxidant defenses in addition to the likely increased production of free radicals as a consequence of the disease. Table 2 shows the plasmatic level of different substances with antioxidant properties. The plasmatic levels of uric acid, bilirubin, transferrin, ascorbate and α-tocopherol did not differ variegate porphyria and control women. Neutrophils produce increased levels of ROS when activated, so their activity can contribute to oxidative stress in the whole organism. We then studied if the porphyric condition affected the oxidative status of neutrophils. Antioxidant defenses and oxidative damage markers in neutrophils are shown in Table 3. VP significantly reduced the activity of the antioxidant enzymes catalase and glutathione peroxidase in neutrophils, while glutathione peroxidase and superoxide dismutase activities were maintained. Intracellular levels of antioxidant vitamins ascorbate and α-tocopherol in neutrophils were not affected by the porphyric condition. Women affected by VP shown increased protein oxidative damage levels in neutrophils, as shown by the increased carbonyl index. Neutrophil priming to oxidative burst was analyzed by ROS production after neutrophil stimulation. As shown in Figure 1, neutrophils from VP patients were more primed to oxidative burst, as shown by the higher ROS production after neutrophil stimulation with zymosan.

With the evidences that variegate porphyria induce a situation of oxidative damage in neutrophils and plasma, we tested the effects of a functional beverage enriched with vitamins C and E on the antioxidant status of porphyric and control subjects. The effects of this nutritional intervention on the antioxidant defenses and the apparition of oxidative damage in plasma and neutrophils are shown in Table 4. The supplementation with the beverage enriched with vitamins C and E for six months significantly attenuated the values of plasma markers for lipid oxidative damage because the

Table 1. Effects of variegate porphyria on serum and plasma damage markers.

	Control	Porphyria
GGT (U/l)	19.8 ± 4.2	28.2 ± 12.1
LDH (U/l)	321 ± 10	313 ± 7
CPK (U/l)	96.4 ± 6.4	88.0 ± 6.4
C-reactive protein (mg/dl)	0.267 ± 0.091	0.567 ± 0.108 #
Plasma MDA (μM)	0.962 ± 0.089	1.31 ± 0.15 #
Plasma carbonyl index (μM)	882 ± 23	873 ± 19

Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups, $p < 0.05$.

Table 2. Effects of variegate porphyria on plasma antioxidant defenses.

	Control	Porphyria
Uric acid (mg/dl)	4.36 ± 0.16	4.34 ± 0.17
Bilirubin (mg/ml)	0.588 ± 0.030	0.530 ± 0.032
Transferrin (mg/dl)	278 ± 12	294 ± 9
Ascorbate (μM)	34.7 ± 3.5	32.9 ± 3.7
α-tocopherol (μM)	26.7 ± 1.1	27.4 ± 1.0

Statistical analysis: Student's t-test for unpaired data. No significant differences were found, $p < 0.05$.

plasma MDA decreased about 30% both in the control and in the porphyric women. However, carbonyl derivatives were not affected by the supplementation. In neutrophils, an effect of the supplementation on the cellular levels of α-tocopherol was observed. Subjects consuming the antioxidant enriched beverage presented higher α-tocopherol levels in neutrophils when compared to subjects consuming the placebo beverage. Ascorbate, MDA and carbonyl levels were not affected in neutrophils by supplementation nor by disease after the nutritional intervention. The antioxidant enzyme activities in neutrophils were not affected by the supplementation (Table 5). The differences between porphyric and control subjects observed in basal conditions concerning catalase and glutathione reductase activities were not evidenced after the supplementation period. Table 5 also shows the activities of antioxidant enzymes in erythrocytes after the diet intervention with antioxidant vitamins. An effect of supplementation was observed on catalase and glutathione reductase activities. Subjects consuming the beverage enriched with vitamins C and E presented higher catalase and glutathione reductase activities in erythrocytes. The difference between the non-enriched and the enriched groups were significant only in the control group, while in the porphyric groups no significant differences were evidenced.

Table 3. Effects of variegate porphyria on neutrophil antioxidant defenses and oxidative damage .

	Control	Porphyria
Catalase (K/10^9 cell)	19.1 ± 1.3	14.9 ± 1.0 #
Glutathione peroxidase (nkat/10^9 cell)	32.4 ± 1.8	29.1 ± 1.9
Glutathione reductase (nkat/10^9 cell)	55.0 ± 4.8	43.0 ± 2.4 #
Superoxide dismutase (pkat/10^9 cell)	19.4 ± 2.3	20.3 ± 1.4
Ascorbate (μM)	798 ± 37	797 ± 34
α-tocopherol (μM)	64.5 ± 10	60.4 ± 6
MDA (μmol/10^9 cell)	1.42 ± 0.27	1.24 ± 0.17
Carbonyl index (μmol/10^9 cell)	27.5 ± 2.2	36.0 ± 3.6 #

Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups, $p < 0.05$.

Discussion

Variegate porphyria is the result of decreased PPOX activity, the penultimate enzyme of heme biosynthesis. This disease is characterized by accumulation of some heme precursors in liver and erythrocytes and by a low rate of heme biosynthesis as a result of the genetic error in PPOX. All the patients participating in our study had been previously diagnosed on the basis of different parameters such as plasma fluorescence peak at 626 nm, levels of excreted urinary and faecal porphyrins and clinical manifestations. It has been shown that the accumulation of the heme precursors constitutes an endogenous source of reactive oxygen species, triggering oxidative damage to cellular components [32, 33]. We studied different damage markers in plasma that are good indexes of cellular damage and global oxidative stress. Gamma glutamyl transpeptidase (GGT) activity has been recently proposed as a good biomarker of whole body stress, with higher GGT activities reflecting a situation of higher oxidative stress [34]. In our study we observed a tendency to higher GGT activities in porphyria patients than their controls, but these porphyric women presented high variability and the difference with respect to controls was not significant. Lactate dehydrogenase (LDH) and creatine kinase (CK) activities are both markers of muscle inflammation and damage [35]. None of these markers were affected by porphyria, suggesting that variegate porphyria does not affect the stability of muscle cells. However, c-reactive protein (CRP) circulating levels were higher in variegate porphyria patients than in control subjects. CRP is a key inflammatory factor produced by the liver in response to inflammation and its concentration in plasma can increase several-fold, so CRP levels are widely used as a marker of chronic inflammation [36]. The higher CRP levels in VP patients can be an indicator of a situation of higher systemic inflammation in these subjects when compared to healthy subjects. In fact, higher CRP levels have been described in porphyria cutanea tarda patients when compared to controls [37]. The presence of higher levels of a lipid oxidative damage marker such as MDA in plasma also evidences a situation of plasma oxidative stress in women affected by VP. This condition of oxidative stress could be a consequence of reduced antioxidant defences in plasma or higher production of ROS. However, none of the antioxidant substances measured in plasma showed differences between porphyric and control subjects. Plasma levels of vitamins C and E did not differ between porphyric and control subjects. In other types of porphyria,

Table 4. Effects of a diet supplementation with vitamins E and C on plasma and neutrophil antioxidant defenses and oxidative damage markers of variegate porphyria patients.

		Control	Porphyria	ANOVA		
				D	S	D*S
Plasma						
MDA	**Non-enriched**	1.07 ± 0.12	0.949 ± 0.110		x	
(μM)	**Enriched**	0.771 ± 0.084	0.677 ± 0.087			
Carbonyl derivatives	**Non-enriched**	2.84 ± 0.20	2.64 ± 0.14			
(mM)	**Enriched**	2.68 ± 0.58	3.15 ± 0.15			
Neutrophils						
Ascorbate	**Non-enriched**	867 ± 77	670 ± 34			
(μM)	**Enriched**	873 ± 70	816 ± 98			
α-tocopherol	**Non-enriched**	51.9 ± 5.7	60.9 ± 3.1		x	
(μM)	**Enriched**	75.8 ± 11.3	81.6 ± 15.1			
MDA	**Non-enriched**	0.648 ± 0.139	0.687 ± 0.086			
($\mu M/10^9$ cell)	**Enriched**	0.581 ± 0.122	0.731 ± 0.158			
Carbonyl derivatives	**Non-enriched**	17.4 ± 1.9	19.5 ± 1.7			
($\mu mol/10^9$ cell)	**Enriched**	18.8 ± 3.0	23.0 ± 3.1			

Statistical analysis: Two-way ANOVA. (D) significant effect of disease; (S) significant effect of supplementation. (DxS) significant interaction between the two ANOVA factors. No significant differences were found, $p < 0.05$.

such as porphyria cutanea tarda, deficiencies in ascorbate, retinol and carotene have been described [4, 5, 38]. However, in variegate porphyria these antioxidant vitamins are not depleted. Other substances with antioxidant properties such as uric acid and bilirubin or the iron binding protein transferrin where not affected either by the porphyric condition. Thus, women affected by variegate porphyria show no sign of alteration in the low molecular antioxidant defences of plasma. However, the increased MDA levels could be an indicator of a situation of oxidative stress in plasma, which could be a direct consequence of the increased heme intermediates or the slightly higher situation of chronic inflammation shown in the porphyric subjects.

Plasma parameters are good markers of the systemic situation, as stated previously. To further corroborate that women affected by variegate porphyria present an oxidative stress condition we studied antioxidant defenses and oxidative damage markers in neutrophils. Neutrophils are related with inflammation processes and they are source of ROS when activated and primed for oxidative burst. In fact, the increased capabilities to produce ROS in neutrophils are related with the pathogenesis of some degenerative diseases such as Alzheimer and Parkinson [39, 40].Women affected by variegate porphyria present lower neutrophil activities of both catalase and glutathione reductase, together with higher ROS production after neutrophil stimulation with opsonized zymosan than control women. Together with the increased values for CRP levels, these results reinforce the situation of chronic inflammation present in variegate porphyria patients. Neutrophils are cells that respond to its stimulation by altering their antioxidant enzyme activities. After intense exercise, neutrophils respond in an acute phase-like immune response, by increasing their number in circulation, decreasing their antioxidant enzyme activities and getting primed to oxidative burst [10]. In these subjects, neutrophils are in a more activated state, with decreased antioxidant enzyme activities and increased susceptibility to activate the oxidative burst. While other leukocytes, such as lymphocytes, usually respond to oxidative stress by increasing enzyme activities [24, 41], neutrophils adapt to oxidative stress (i.e. induced by exercise) by reducing antioxidant enzyme activities [10, 41]. Thus, women affected by variegate porphyria have primed neutrophils to oxidative burst. Furthermore, we found evidences of protein oxidative damage in neutrophils of variegate porphyria women. δ-aminolevulinic acid has been shown to directly oxidize proteins [42, 43], so the increased levels of oxidized groups in the proteins from VP patients could be a direct effect of heme precursors over them. The decreased catalase activity shown by women affected by variegate porphyria can therefore be attributed to three different mechanisms: (1) As catalase has heme as a prosthetic group, the limitation in heme synthesis

Table 5. Effects of a diet supplementation with vitamins E and C on neutrophil and erythrocyte antioxidant enzyme activities of variegate porphyria patients.

		Control	Porphyria	ANOVA		
				D	S	D*S
Neutrophils						
Catalase ($K/10^9$ cell)	**Non-enriched**	24.2 ± 2.3	30.6 ± 3.5			
	Enriched	28.3 ± 3.2	33.5 ± 5.5			
Glutathione peroxidase (nkat/10^9 cell)	**Non-enriched**	49.2 ± 8.2	58.5 ± 6.8			
	Enriched	59.8 ± 9.6	51.8 ± 7.6			
Glutathione reductase (nkat/10^9 cell)	**Non-enriched**	35.5 ± 4.2	34.3 ± 9.5			
	Enriched	31.1 ± 4.4	32.7 ± 4.4			
Superoxide dismutase (pkat/10^9 cell)	**Non-enriched**	28.7 ± 7.2	40.1 ± 8.1			
	Enriched	39.3 ± 6.1	42.9 ± 10.8			
Eryhtocytes						
Catalase ($K/10^6$ cell)	**Non-enriched**	6.76 ± 0.35	7.23 ± 0.42			
	Enriched	10.0 ± 1.4 §	8.02 ± 0.77		x	
Glutathione peroxidase (nkat/10^9 cell)	**Non-enriched**	14.7 ± 0.8	19.8 ± 2.4			
	Enriched	17.8 ± 1.9	21.5 ± 2.6			
Glutathione reductase (nkat/10^9 cell)	**Non-enriched**	3.91 ± 0.35	3.87 ± 0.40			
	Enriched	5.83 ± 0.51§	4.20 ± 0.17		x	
Superoxide dismutase (pkat/10^9 cell)	**Non-enriched**	3.27 ± 0.54	4.25 ± 0.52			
	Enriched	4.23 ± 0.41	4.40 ± 0.71			

Statistical analysis: Two-way ANOVA. (D) significant effect of disease; (S) significant effect of supplementation. (DxS) significant interaction between the two ANOVA factors. (§) indicates significant differences between non-enriched and enriched beverage groups, $p < 0.05$.

attributed to the genetic defect in PPOX could lead to limitations in the synthesis of active catalase, thus reducing total enzyme activity, (2) the increased levels of d-ALA or other ROS, as a consequence of the accumulation of heme precursors in VP patients, could directly oxidize catalase protein causing oxidative damage and reducing enzyme activity, or (3) the activation state of neutrophils as a consequence of the disease induce the catalase release to plasma as it has been pointed out previously [10]. The proposals (2) and (3) can also be applied to the observed decrease in glutathione reductase.

Once evidenced that women affected by variegate porphyria show increased oxidative stress than control healthy women, we tested the effects of a diet supplementation with an almond based beverage enriched with vitamins C and E. The diet supplementation with vitamin E has been shown to be effective in reducing porphyrin levels in patients affected by porphyria cutanea tarda [44-46]. However, few studies have been performed concerning the effects of an antioxidant supplementation on variegate porphyria patients, and these studies have been focused on the effects on symptoms or porphyrin excretion rather than in the oxidative stress condition of patients [47, 48]. In our double-blinded cross-over study, porphyric and healthy women were supplemented with vitamins C and E (150mg/d and 50mg/d, respectively) during six months, using an almond based beverage as the carrier and placebo. The diet supplementation with the enriched drink induced lower levels of plasma MDA. The intracellular neutrophil levels of α-tocopherol were increased in the supplemented groups, although no effects of supplementation were observed on oxidative damage markers and antioxidant enzyme activities in neutrophils. The differences in antioxidant enzyme activities and carbonyl derivatives between porphyric and control subjects described in basal conditions were not evidenced after the supplementation period in the placebo nor in the supplemented groups, thus indicating that the almond beverage used as the vehicle for supplementation could have had beneficial effects on these parameters. However, the lack of a negative control group without the beverage intake makes difficult to conclude that.

We finally studied the effects of the antioxidant supplementation on the erythrocyte activities of antioxidant enzymes. The diet supplementation with vitamins C and E induced the activity of catalase and

glutathione reductase in erythrocytes. As erythrocytes can not express genes nor synthesize new proteins, these results indicate that changes in glutathione reductase and superoxide dismutase activities can be attributed to direct effects on the protein. Glutathione reductase and superoxide dismutase can be directly activated by transcriptional events [49, 50]. In addition, we have previously evidenced in an *in vitro* experiment that glutathione reductase activity increases when it is measured in the presence of catalase [49]. It is therefore evident that the activity of antioxidant enzymes can be directly modified by the levels of ROS. The higher amounts of antioxidants could protect the catalytic site or stability of glutathione reductase and catalase. Furthermore, a synergistic effect between catalase and glutathione reductase could be implicated in the activation of these enzymes.

In conclusion, women affected by variegate porphyria present a situation of chronic inflammation and plasma oxidative damage. Neutrophils from variegate porphyria women are in a more activated state than those from healthy women, with decreased catalase and glutathione reductase activities and higher reactive oxygen species production and protein oxidative damage. The diet supplementation with vitamin E (50 mg/day) and vitamin C (150 mg/day) during six months decreases plasma oxidative damage and enhances the erythrocyte activities of catalase and glutathione reductase.

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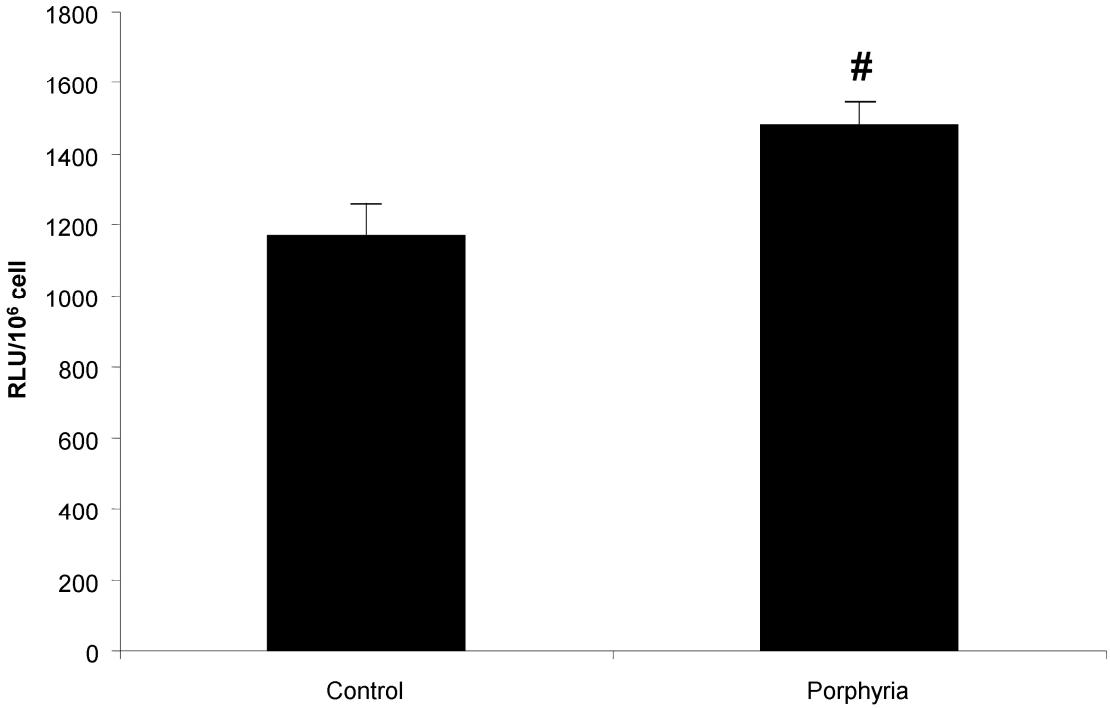
FIGURES

Figure 1. Effects of variegate porphyria on neutrophil ROS production after stimulation with opsonized zymosan.



Statistical analysis: Student's t-test for unpaired data. (#) indicates significant differences between porphyria and control groups ($p < 0.05$).

Manuscript XIV

Phytoestrogens enhance antioxidant enzyme defenses and reduce testosterone and estradiol circulating levels.

Mestre A, Ferrer MD, Sureda A, Tauler P, Martínez E, Bibiloni M, Micol V, Tur JA, Pons A.

Phytoestrogens enhance antioxidant enzyme defenses and reduce testosterone and estradiol circulating levels

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Abstract
Our aim was to evidence the effects of a diet supplementation with phytoestrogens on the antioxidant adaptative responses, sexual hormone levels and oxidative damage induced by moderate exercise. Ten female swimmers participated for 26 days in a double-blinded diet intervention with either a functional beverage rich in vitamins E and C or the same beverage but also supplemented with *Lippia citriodora* extract (PLx). After the intervention all subjects participated in a swimming session. Swimming increased the erythrocyte activity of glutathione peroxidase and glutathione reductase only in the PLx-supplemented. In lymphocytes, the superoxide dismutase activity increased after exercise in both supplemented groups, but the increase was higher in the group supplemented with PLx. The gene expression of Mn-SOD, as well as Bcl-2 and UCP-3, was however maintained in both groups after exercise. No effects were observed in the lymphocyte levels of MDA and carbonyls, but exercise increased the percentage of high-damaged lymphocytes about 2.8 times in the placebo group and 1.5 times in the PLx-supplemented group. PLx decreased the levels of 17-β-estradiol and testosterone and increased the levels of the sexual hormone binding protein. In summary, the diet supplementation with phytoestrogens such as verbascoside and martynoside decrease the number of circulating erythrocytes and leukocytes and enhances the glutathione-dependent enzyme activities in erythrocytes and the superoxide dismutase activity in lymphocytes in response to exercise. The diet supplementation with verbascoside and martynoside also decreases the plasma steroid hormone levels, pointing out a possible agonist effect between estradiol and these phytoestrogens.

Introduction

The reactive oxygen species (ROS) are a double-edge sword because they serve as key signaling molecules in physiological processes [1-3] but they also have a role in pathological processes as those involving the female reproductive tract [4, 5] or the upper respiratory tract diseases associated to exercise [6] or aging [7]. Both exhaustive and moderate exercise may increase ROS production [8, 9] but also the antioxidant capability as occurs in lymphocytes [10, 11]. ROS production runs parallel to the activation of antioxidant defenses and a correlation exists between the lymphocyte antioxidant enzyme activities and the markers of oxidative damage after exercise [12]. The ROS and the antioxidants remain in balance in a healthy body, but it is a dynamic balance because an increase in ROS production has to be compensated with an increase in the expression of antioxidant enzymes. A relative delay exists between the ROS production and the adaptative response to synthesize new antioxidant enzymes and then, other more immediate antioxidants systems could operate, such as the direct ROS deactivation by low molecular weight antioxidants and the constitutive antioxidant enzymes. Thus, increased levels of ROS induce oxidative damage but also the expression of antioxidant enzymes [10, 13]. The diet supplementation with antioxidant nutrients could eliminate the endogenous activation of antioxidant defenses because of the direct deactivation of ROS by the exogenous antioxidants. In fact some diet supplementation studies with antioxidant nutrients attenuates the activation of antioxidant enzymes such as catalase and glutathione peroxidase [14, 15]. Therefore, the dosage of antioxidant nutrients is crucial in order to avoid oxidative damage and, at the same time, maintain or enhance the antioxidant endogenous defenses. The diet supplementation with an almond based beverage enriched with vitamins E and C for 1 month has been evidenced as useful to avoid the oxidative damage without intercepting the adaptative response to exercise [13]. Higher doses of

antioxidants could alter this response, and then new studies are needed to evaluate the effects of a higher antioxidant capability of this beverage on the oxidative damage and antioxidant response to exercise. The *Lippia citriodora* extract (PLx) contains the phenylpropanoid glycosides verbascoside and martynoside as the main compounds and other phytosterols. Verbascoside and martynoside are phenylpropanoid glycosidic compounds with antioxidant activities [16-20]. They are widely distributed in the plant kingdom and are major components of the phenolic fraction of various plant extracts [21, 22]. The aforementioned compounds exhibit antiproliferative, cytotoxic, antimetastatic and immunomodulatory properties [23-26] but may also act as estrogen agonists/antagonists via the known estrogen receptor alpha (ERα) and beta (ERβ) subtypes [27-29]. The introduction of PLx extract to an almond based beverage in order to obtain a functional food will produce a beverage with higher antioxidant potential but also with estrogenic activity.

The antioxidant nutrients, in addition to their capability to react with ROS, can also directly induce the expression of antioxidant enzymes [1]. The antioxidants can modulate the expression of antioxidant genes through the interaction of receptor complexes with the estrogen response element (ERE) and/or the antioxidant response element (ARE) in the promoter region of target genes [30, 31]. The estrogen receptor, a nuclear steroid receptor, binds estrogens and regulates the transcription of estrogen-responsive genes by interacting directly with DNA at particular estrogen response elements (ERE) of their promoters, or with other transcription factors, such as AP-1 or NF-κB bound to their cognate DNA sequences [32]. Phytoestrogens are natural estrogenic compounds and antioxidant nutrients present in foods as soy, almond, orange, medicinal plants, etc. The phytoestrogens, in addition to their capability to react with ROS, can also induce the antioxidant gene expression by their interaction with estrogen receptors and the NF-κB-signaling pathway, independent of ERE or ARE nuclear elements [33]. In fact, the dietary soy phytoestrogens improve endothelial function, increase eNOS gene expression and reduce blood pressure in rats [31]. However, the effects of phytoestrogens on the estrogen production have not been studied until now.

Marked differences exist in the oxidant production between males and females [34, 35], and these differences have been attributed to the testosterone production in males and the estradiol production in females [36, 37]. It has been suggested that testosterone stimulates total body oxidative estress while estrogens decrease total body oxidative stress [38]. The intake of phytoestrogens could bring to males the advantages of females against the oxidative stress without the risk of feminizing. However, the effects of phytoestrogens on the sexual hormone levels need to be studied because several studies of estrogenic compounds present in the environment indicate the existence of effects on the behavior [39] and on the reproductive system of animals [40].

We hypothesize that the diet supplementation with antioxidant phytosterols could affect the antioxidant response to exercise and alter the sexual hormone production in female swimmers. Our aim was to evidence the effects of a diet supplementation with phytoestrogens on the antioxidant adaptative responses, sexual hormone levels and oxidative damage induced by moderate exercise in well nourished and trained swimmers.

Materials and methods

Participants and protocol

Ten voluntary girls (16.1 ± 2.2 years) participated in this study. They all were swimmers belonging to amateur teams. Participants and their parents were informed of the purpose of this study and the possible risks involved before parents gave their written and informed consent to the minor taking part in the study. The study protocol was in accordance with the Declaration of Helsinki and was approved by the Balearic Islands Clinical Investigations bioethics committee.

The subjects were randomly and double-blind treated with either a functional beverage rich in vitamins E and C (10 and 30 mg/100 ml, respectively) or the same functional food but additionally supplemented with 400 mg/100 ml of *Lippia citriodora* extract. The *Lippia citriodora* extract (Planox L, PLx) contains 10% verbascoside and 5-8% other phenylpropanoids such as martynoside and isoverbascoside, so the PLx supplement contained about 40 mg/100 ml verbascoside. All participants drank 500 ml/day of the beverage for 26 days.

After the nutritional intervention all subjects participated in a habitual training session of swimming. The exercise session was as follows: Participants warmed up for 30 minutes prior to starting the exercise protocol. The participants performed the exercise session by series of 50 m swims for 30 minutes maintaining the intensity about 80% of their maximal capacity. Swimmers

were at rest for about 10-15 seconds between each swim. Previous tests were performed to allow the individual design of the protocol and to ensure that all the swimmers would be able to complete the exercise protocol. The mean of individual best marks in 50 m was 32.3 ± 1.1 s in the placebo group and 32.3 ± 0.9 s in the PLx group. The mean speed at 80% of own maximal mark was 1.29 ± 0.04 m/s in the placebo group and 1.29 ± 0.03 m/s in the PLx group. The swimming session supposed a modest intensity workout for the participants in the study. Blood samples were obtained before (in basal conditions after overnight fasting) and 1 hour after swimming because changes in antioxidant enzymes and appearance of oxidative damage become evident 1 hour after finishing the exercise bout rather than immediately after [10, 41]. Previous to the swimming session all participants ate a controlled meal (796Kcal; 24.7g of proteins; 145g carbohydrates; 36.5g lipids; 4.00g fiber) and immediately after exercise all participants drank 200mL of their respective almond based beverage.

Analytical HPLC

Lippia citriodora hydroalcoholic extract was analyzed by using HPLC. Powered extract was dissolved at 1 mg/ml in distilled water and centrifuged for 20 min at 150g. 10 µl of the supernatant were injected in an analytical reverse phase column LiChrospher® 100 RP-18 (5 µm, 250 x 4 mm i.d.) from Merck and subjected to HPLC analysis. The separation of the compounds was carried out in a high performance liquid chromatography LaChrom (Merck-Hitachi) series 7000 system, equipped with a pump, autosampler, column oven and UV-Vis diode array detector (wavelength selected at 340 nm to detect the phenylpropanoids). The chromatographic separation was performed at 25ºC and a flow rate of 1 ml/min was used with a mobile phase composed of (A) ACN: Phosphoric acid 0.45N (1:9) and (B) ACN: Phosphoric acid 0.45N (9:1). The multigradient solvent system was as follows: 0-25 min, from 0% to 12% B; 25-45 min, from 12% to 20% B; 45-47 min from 20% to 60% B; 47-56 min, fixed at 60% B; 56-57 min from 60% to 0% B and 13 min more for reequilibration at 0% B.

The linearity range of the responses for the standard was determined on eight concentration levels by triplicate. Calibration graphs for HPLC were recorded with sample amount ranging from 0.25 µg/ml to 0.25 mg/ml (r^2>0.9999). Quantitative evaluation of verbascoside was performed by means of a six-point regression curve (r^2>0.996) in a concentration range between 0 and 0.1 mg/ml, using verbascoside as reference external standard and evaluated at 340 nm. LOD (Limit of Detection) was 0.1 µg/ml and LOQ (Limit of Quantification) was 0.25 µg/ml.

Experimental procedure

Venous blood samples were obtained from the antecubital vein of swimmers in suitable vacutainers with EDTA as anticoagulant. Leukocyte counts and hematological parameters were determined on whole blood. Progesterone, 17-b-estradiol, testosterone, free testosterone and SHBG levels were determined in plasma. Lymphocyte and erythrocytes fractions were purified. Catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase activities were determined in erythrocytes and lymphocytes. Malondialdehyde, protein carbonyl index and DNA damage were measured in lymphocytes. The expression of catalase, glutathione peroxidase, Mn-superoxide dismutase, Bcl-2, UCP3 and hemeoxygenase 1 were assessed in lymphocytes.

Anthropometric data

The anthropometric variables measured in this study were height, body mass, triceps skinfold thickness, mid-upper-arm circumference, and body composition. Height was determined using a mobile anthropometer (Kawe 44444, France) to the nearest millimetre, with the participant's head in the Frankfurt plane. Body mass was determined to the nearest 100 g using a digital scale (Tefal, sc 9210, France). Participants were weighed barefoot and while wearing light underwear, which was accounted for by subtracting 300 g from the measured weight. Brachial, waist, hip and thigh perimeters were measured to the nearest 0.1 cm with the participant's right arm relaxed, using a non-stretchable measuring tape (KaWe, 43972, France). Percent body fat and mass of body fat were calculated by bioimpedance using a hand-held BIA unit (Omron1BF 300 body fat monitor). All anthropometric measurements were performed by one observer to avoid inter-observer variation.

Different anthropometric indexes were calculated using these measurements: body mass index [BMI = mass (kg)/squared height (m)]; Index waist-hip [waist perimeter (cm)/hip perimeter (cm)].

Dietary intake

Dietary habits were assessed using a 3-day dietary record questionnaire completed at the beginning and in the week before the exercise test. A qualified dietician verified and quantified the food records. All food items consumed were transformed into nutrients using a special computerized program based on the European [42] and Spanish [43, 44] food composition tables.

Clinical determinations

Leukocyte counts and hematological parameters such as erythrocyte number, haemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and coefficient of variation of the red cell width (RDW) were determined in an automatic flow cytometer analyzer Technicon H2 (Bayer) VCS system.

Progesterone, 17-b-estradiol, testosterone and free testosterone levels were determined by standard laboratory procedures using a direct chemiluminescence immunoassay [45] and an automatic analyzer Centauro (Siemens). SHBG levels were determined by standard laboratory procedures using an immunochimioluminescent analyzer Immulite (Siemens).

Lymphocyte and erythrocyte purification

Blood samples were processed following an adaptation of the method described by Boyum [46]. Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and was then centrifuged at 900xg, at 18ºC for 30 min. The lymphocyte layer was then carefully removed, washed twice with PBS and centrifuged for 10 minutes at 1000xg, 4ºC. The cellular precipitate of lymphocytes was lysed with distilled water.

For erythrocyte purification, blood was centrifuged at 900xg, at 4ºC for 30 min. The erythrocyte phase at the bottom was washed twice with 10 ml of PBS and was finally reconstituted with distilled water in the same volume as plasma. Then the erythrocyte resuspension was haemolysed with distilled water (1:1).

mRNA gene expression

mRNA expressions in lymphocytes were determined by real time RT-PCR with 18S ribosomal as reference gene. For this purpose, mRNA was isolated from lymphocytes by extraction with Tripure (Roche). cDNA was synthesized from 1 μg total RNA using reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. The primers used are shown in Table 1. For all PCRs there was one cycle at 95ºC for 10 min, followed by 40 cycles at the conditions shown in Table 1.

The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Basal mRNA levels at the beginning of the stage were arbitrarily referred to as 1. The expression of the target gene was normalized with respect to ribosomal 18S.

Table 1. Primers and conditions used in Real Time PCRs

Gene	Primers	Conditions
18S	Fw: 5'-ATG TGA AGT CAC TGT GCC AG-3' Rv: 5'-GTG TAA TCC GTC TCC ACA GA-3'	95ºC, 10 s; 60ºC, 7 s; 72ºC, 12 s
Catalase	Fw: 5'-TTT GGC TAC TTT GAG GTC AC-3' Rv: 5'-TCC CCA TTT GCA TTA ACC AG-3'	95ºC, 10 s; 60ºC, 10 s; 72ºC, 15 s
GPx	Fw: 5'-TTC CCG TGC AAC CAG TTT G-3' Rv: 5'-TTC ACC TCG CAC TTC TCG AA-3'	94ºC, 1 s; 60ºC, 7 s; 72ºC, 5 s
Bcl-2	Fw: 5'-CTG GTG GGA GCT TGC ATC AC-3' Rv: 5'-ACA GCC TGC AGC TTT GTT TC-3'	95°C, 5 s; 65ºC, 5 s; 72ºC, 5 s
UCP-3	Fw: 5'- CGTGGTGATGTTCATAACCTATG-3' Rv: 5'- CGGTGATTCCCGTAACATCTG-3'	95°C, 5 s; 60ºC, 7 s; 72ºC, 10 s
MnSOD	Fw: 5'-GAG AAG TAC CAG GAG GCG TTG-3' Rv: 5'- CAA GCC AAC CCC AAC CTG AGC-3'	95ºC, 30 s; 64ºC, 60 s
HO-1	Fw: 5'-CCA GCG GGC CAG CAA CAA AGT GC-3' Rv: 5'-AAG CCT TCA GTG CCC ACG GTA AGG-3'	95ºC, 0 s; 60ºC, 5 s; 72ºC, 10s

Enzymatic determinations

All activities were determined in lymphocytes and eryhtrocytes with a Shimadzu UV-2100 spectrophotometer at 37ºC. Catalase (CAT) activity was measured by the spectrophotometric method of Aebi based on the decomposition of H_2O_2 [47]. Glutathione peroxidase (GPx) activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler [48] using H_2O_2 as substrate. Glutathione reductase (GR) activity was measured by a modification of the Goldberg and Spooner spectrophotometric method [49]. Superoxide dismutase (SOD) activity was measured by an adaptation of the method of McCord and Fridovich [50].The xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion produced the reduction of cytochrome *c*, which was monitored at 550 nm. The superoxide dismutase in the sample removed the superoxide anion and produced an inhibition of the cytochrome *c* reduction.

Malondialdehyde determination

Malondialdehyde (MDA) as a marker of lipid peroxidation was analyzed in lymphocytes by a colorimetric assay kit (Calbiochem, San Diego, CA, USA). The method used is specific for malondialdehyde determination

Protein carbonyl determination

Protein carbonyl derivatives were determined in lymphocytes by an immunological method using the OxyBlotTM Protein Oxidation Detection Kit (Chemicon International) following manufacturer's details. Briefly, 10 µg of protein were incubated in the presence of 2,4-dinitrophenylhydrazine (DNPH). Once derivatized, samples were transferred to a nitrocellulose membrane by the method of dot blot. The membrane was then incubated with primary antibody, specific to the DNP moiety of the proteins. This step was followed by incubation with a horseradish peroxidase-antibody (goat anti-rabbit IgG) conjugate directed against the primary antibody. The membranes were then treated with luminol, which is converted to a light-emitting form at wavelength 428 nm by the antigen/primary antibody/secondary antibody/peroxidase complex. The light was visualized and detected by short exposure to a Molecular Imager Chemidoc XRS (Bio-Rad Laboratories). Image analysis was performed using Quantity One-1D analysis software (Bio-Rad Laboratories).

Comet assay

Assessment of DNA damage was carried out in lymphocytes using the comet assay method. Briefly, slides were prepared by adding 20 ml purified lymphocytes, mixed with 80 ml 0.6% low-melting-point agarose. In order to release the DNA, cells were lysed by immersing slides in lysing solution (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mM Tris, 1% Triton X-100 and 10% DMSO added freshly, pH 10) at 4ºC. DNA was denatured by placing slides in alkaline bath in the electrophoresis tank for 40 min to allow for the unwinding of the DNA and expression of alkali-labile damage. Electrophoresis was carried out with an electric current of 250 mV applied for 30 min. Tris buffer pH 7.5 was added onto the slides to neutralize excess alkali. Finally, DNA was stained by adding 80 ml ethidium bromide (20 mg/ml) to each slide.

Comet measurements were made by image analysis using a fluorescence microscope and the Comet software (TriTek CometScore™, version 1.5). Images of 100 randomly selected nuclei were analyzed for each sample

Table 2. Anthropometric evaluation in women swimmers at the beginning and the end of the nutritional intervention

		Beginning	End	ANOVA S	I	SxI
Age (years)	**Placebo**	15,3 ± 0,5	15,3 ± 0,5			
	PLx	16,8 ± 3,1	16,8 ± 3,1			
Weight (kg)	**Placebo**	60,9 ± 4,4	58,2 ± 2,4			
	PLx	58,2 ± 11,4	58,1 ± 11,0			
Height (cm)	**Placebo**	167 ± 6	168 ± 6			
	PLx	168 ± 8	168 ± 8			
IMC (kg/m^2)	**Placebo**	21,8 ± 1,6	20,7 ± 1,1			
	PLx	20,3 ± 1,9	20,4 ± 1,8			
Brachial perimeter (cm)	**Placebo**	26,5 ± 1,2	26,1 ± 0,7			
	PLx	25,9 ± 2,4	25,6 ± 1,9			
Waist perimeter (cm)	**Placebo**	70,9 ± 3,9	68,2 ± 2,9			
	PLx	67,6 ± 4,9	65,9 ± 5,4			
Hip perimeter (cm)	**Placebo**	92,9 ± 4,1	89,1 ± 1,7			
	PLx	92,2 ± 7,7	91,8 ± 6,9			
Index waist-hip	**Placebo**	0,76 ± 0,04	0,77 ± 0,04			
	PLx	0,73 ± 0,02	0,72 ± 0,03 *	x		
Thigh perimeter (cm)	**Placebo**	49,3 ± 1,2	48,8 ± 1,0			
	PLx	47,5 ± 4,4	47,5 ± 3,8			
Corporal density (kg/m^3)	**Placebo**	1,05 ± 0,01	1,06 ± 0,01			
	PLx	1,06 ± 0,01	1,06 ± 0,01			
% Fat body mass	**Placebo**	20,8 ± 3,2	19,9 ± 1,7			
	PLx	18,9 ± 3,3	19,0 ± 3,3			

Statistical analysis: Two-way ANOVA. (S) Significant effect of supplementation, (I) Significant effect of the diet intervention. (SxI) significant interaction between both factors. (*) Significant differences between Placebo and PLx, $p<0.05$.

Table 3. Intake of macronutrients and micronutrients (minerals and vitamins) in women swimmers at the beginning and the end of the nutritional intervention.

		Beginning	End	ANOVA S	I	SxI
Energy (Kcal)	**Placebo**	2431 ± 295	3098 ± 325 #			
	PLx	2459 ± 577	2776 ± 680		x	
Proteins (g)	**Placebo**	110 ± 20	127 ± 15			
	PLx	124 ± 3	110 ± 31			
Carbohydrates (g)	**Placebo**	311 ± 55	382 ± 51			
	PLx	351 ± 5,0	331 ± 84			
Fats (g)	**Placebo**	122 ± 30	126 ± 28			
	PLx	151 ± 5	129 ± 33			
Cholesterol (mg)	**Placebo**	463 ± 116	373 ± 64			
	PLx	549 ± 26	360 ± 94 #		x	
Thiamin (mg)	**Placebo**	3,50 ± 1,25	2,1 ± 0,4			
	PLx	3,46 ± 0,19	3,3 ± 3,3			
Riboflavin (mg)	**Placebo**	4,63 ± 0,66	2,6 ± 0,7 #			
	PLx	4,54 ± 0,25	4,3 ± 4,6			
Vitamina B_6 (mg)	**Placebo**	3,29 ± 0,58	3,6 ± 0,8			
	PLx	3,30 ± 0,06	2,8 ± 1,0			
Vitamin B_{12} (µg)	**Placebo**	13,5 ± 6,8	21,7 ± 9,5			
	PLx	17,1 ± 1,1	14,9 ± 4,8			
Vitamin C (mg)	**Placebo**	133 ± 25	306 ± 46 #			
	PLx	132 ± 4	297 ± 76 #		x	
Vitamin D (µg)	**Placebo**	4,10 ± 1,23	4,6 ± 3,8			
	PLx	3,80 ± 0,25	4,4 ± 3,2			
Vitamin E (mg)	**Placebo**	14,4 ± 6,55	60,9 ± 3,8 #			
	PLx	21,3 ± 1,62 *	59,5 ±3,0 #		x	x
Niacin (mg)	**Placebo**	33,8 ± 8,6	41,9 ± 6,7			
	PLx	36,1 ± 0,04	31,6 ± 10,7			
Pantothenic acid (mg)	**Placebo**	7,94 ± 1,66	10,2 ± 4,2			
	PLx	7,98 ± 0,14	7,3 ± 1,2			
Folic Acid (µg)	**Placebo**	428 ± 91	462 ± 108			
	PLx	413 ± 1,5	368 ± 148			

Statistical analysis: Two-way ANOVA. (S) Significant effect of supplementation, (I) Significant effect of the diet intervention. (SxI) significant interaction between both factors. (*) Significant differences between Placebo and PLx. (#) Significant differences between the beginning and the end of the nutritional intervention, $p<0.05$.

Statistical analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 13.0 for Windows). Results are expressed as mean ± s.e.m. and $P < 0.05$ was considered statistically significant. A Kolmogorov-Smirnov test was applied to assess that data followed a normal distribution. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analyzed were (I) nutritional intervention, (E) exercise and (S) supplementation. When significant effects were found, a one-way ANOVA was used to determine the differences between the groups involved.

Results

The composition of the hydroalcoholic *Lippia citriodora* extract used in this nutritional intervention was analyzed by means of HPLC-diode array. The identification of the major peaks was based on the analysis of their retention time and UV spectra by comparison with those of authentic standards or data previously reported in the literature. Figure 1 shows the HPLC profile at 340 nm of *Lippia citriodora* extract in which verbascoside and its isomer isoverbascoside were identified as the major components [51]. Some other minor compounds appearing in the chromatogram were identified as glycosylated flavanones, probably glucuronidated derivatives of luteolin as it has been previously reported [52]. The amount of verbascoside in the extract was determined as 10 ± 0.5 % (w/w), using a commercially available standard.

Table 4. Effects of a swimming session and a supplementation with *Lippia citriodora* extract (PLx) on the leukocyte counts and hematological parameters.

		Before	After	ANOVA E	S	ExS
Erythrocytes (10^6/µl)	**Placebo**	4,80 ± 0,08	4,69 ± 0,04			
	PLx	4,49 ± 0,12 #	4,32 ± 0,09 #		x	
Haemoglobin (g/dl)	**Placebo**	14,1 ± 0,4	13,8 ± 0,3			
	PLx	13,3 ± 0,5	12,8 ± 0,3		x	
Hematocrit (%)	**Placebo**	42,3 ± 0,8	41,1 ± 0,5			
	PLx	40,0 ± 0,9	38,2 ± 0,6 #		x	
MCV (fL)	**Placebo**	88,1 ± 0,8	87,8 ± 0,8			
	PLx	89,1 ± 0,6	88,4 ± 0,5			
MCH (pg)	**Placebo**	29,4 ± 0,4	29,4 ± 0,4			
	PLx	29,6 ± 0,2	29,6 ± 0,2			
MCHC (g/dl)	**Placebo**	33,3 ± 0,4	33,5 ± 0,4			
	PLx	33,2 ± 0,5	33,5 ± 0,4			
RDW-SD (fL)	**Placebo**	40,8 ± 1,0	40,4 ± 1,1			
	PLx	42,1 ± 0,9	41,7 ± 0,7			
RDW-CV (%)	**Placebo**	12,7 ± 0,3	12,6 ± 0,3			
	PLx	12,9 ± 0,2	12,9 ± 0,2			
Leukocytes (10^3/ml)	**Placebo**	5,72 ± 0,44	6,51 ± 0,50			
	PLx	4,74 ± 0,49	5,39 ± 0,44		x	
Neutrophil (10^3/µl)	**Placebo**	3,04 ± 0,26	4,14 ± 0,42			
	PLx	2,42 ± 0,48	3,39 ± 0,39	x		
Lymphocytes (10^3/µl)	**Placebo**	2,00 ± 0,18	1,68 ± 0,09			
	PLx	1,81 ± 0,15	1,49 ± 0,15			
Monocyte (10^3/µl)	**Placebo**	0,458 ± 0,056	0,512 ± 0,059			
	PLx	0,357 ± 0,050	0,377 ± 0,046			
Eosinophil (10^3/µl)	**Placebo**	0,192 ± 0,077	0,142 ± 0,069			
	PLx	0,140 ± 0,032	0,110 ± 0,031			
Basophil (10^3/µl)	**Placebo**	0,032 ± 0,015	0,030 ± 0,013			
	PLx	0,015 ± 0,003	0,013 ± 0,002			

Statistical analysis: Two-way ANOVA. (E) significant effect of exercise; (S) significant effect of supplementation. (ExS) significant interaction between the two ANOVA factors. (#) significant differences between placebo and PLx supplementation, $p<0.05$.

The anthropometric characteristics of all the young women swimmers participating in the study are shown in Table 2. No effects were observed concerning the diet supplementation with PLx extracts during 26 days on the anthropometric characteristics. Table 3 shows the nutritional intake by the subjects before and after the nutritional intervention. No differences were observed in the nutrient intake between the placebo and PLx supplemented groups, although the nutrient intake reflected the introduction of an energetic drink rich in antioxidant vitamins E and C in the nutritional habits as a supplement. During the diet intervention the energy intake increased as well as the intake of vitamins E and C, while the intake of cholesterol decreased. No differences were evidenced in the nutrient intake between the placebo and PLx supplemented groups at the end of the diet intervention. The only difference was that the PLx supplemented group intook 2g/d of PLx extract (200mg/d of verbascoside and other phenylpropanoid glycosides in a minor quantity) whereas the placebo group did not.

The diet supplementation with PLx affected some erythrocyte and leukocyte parameters (Table 4). The group supplemented with PLx presented about 7% lower basal erythrocyte counts, haemoglobin and hematocrit, and about 17% lower leukocyte counts than the group supplemented without PLx. The exercise performed by the swimmers for 30 minutes at 80% of its maximal record practically maintained the same basal values of the parameters that characterize erythrocytes such as MCV, MCH, MCHC, RDW-SD and RDW-CV and also maintained the same basal values of lymphocytes, monocytes, eosinophils and basophils. Only the neutrophils were significantly affected by exercise in a similar way in the two supplemented groups, by increasing about 30% after exercise.

The diet supplementation with PLx affected the activity of some antioxidant enzymes in erythrocytes (glutathione peroxidase and glutathione reductase), but not in lymphocytes (Table 5). In the same way the exercise significantly affected the activities of both glutathione dependent antioxidant enzymes in erythrocytes and the superoxide dismutase activity in lymphocytes. A significant interaction between exercise and supplementation was observed in the activity of glutathione reductase in erythrocytes. Exercise significantly increased the erythrocyte activity of glutathione peroxidase (about 22%) and glutathione reductase (about 25%) only in the swimmers that consumed the supplement with PLx, while the swimmers that intook the placebo

Table 5. Effects of a swimming session and a supplementation with *Lippia citriodora* extract (PLx) on lymphocyte and erythrocyte antioxidant enzyme activities.

		Before	After	ANOVA		
				E	S	ExS
Erythrocytes						
Catalase	**Placebo**	4.80 ± 0.34	4.98 ± 0.29			
(K/10^9 cell)	**PLx**	4.48 ± 0.53	5.72 ± 0.53			
Glutathione peroxidase	**Placebo**	35.1 ± 0.6	38.2 ± 0.8			
(nkat/10^9 cell)	**PLx**	37.6 ± 0.7	45.7 ± 3.7* #	x	x	
Glutathione reductase	**Placebo**	1.13 ± 0.04	1.15 ± 0.03			
(nkat/10^9 cell)	**PLx**	1.10 ± 0.04	1.37 ± 0.03* #	x	x	x
Superoxide dismutase	**Placebo**	1.28 ± 0.06	1.37 ± 0.12			
(pkat/10^9 cell)	**PLx**	1.28 ± 0.02	1.28 ± 0.11			
Lymphocytes						
Catalase	**Placebo**	10.8 ± 1.3	13.2 ± 1.1			
(K/10^9 cell)	**PLx**	13.8 ± 2.9	16.7 ± 2.4			
Glutathione peroxidase	**Placebo**	157 ± 12	182 ± 10			
(nkat/10^9 cell)	**PLx**	167 ± 17	190 ± 20			
Glutathione reductase	**Placebo**	66.2 ± 6.7	67.0 ± 4.2			
(nkat/10^9 cell)	**PLx**	57.2 ± 8.5	74.5 ± 9.8			
Superoxide dismutase	**Placebo**	22.3 ± 1.6	29.4 ± 4.0			
(pkat/10^9 cell)	**PLx**	22.6 ± 5.1	34.1 ± 5.8	x		

Statistical analysis: Two-way ANOVA. (E) significant effect of exercise; (S) significant effect of supplementation. (ExS) significant interaction between the two ANOVA factors. (*) significant differences between before and after the swimming session. (#) significant differences between placebo and PLx supplementation, $p<0.05$.

Table 6. Effects of a swimming session and a supplementation with *Lippia citriodora* extract (PLx) on lymphocyte gene expression.

		Before	After	ANOVA E	S	ExS
Catalase	**Placebo**	0.950 ± 0.151	1.05 ± 0.16			
	PLx	1.17 ± 0.16	1.32 ± 0.34			
GPx	**Placebo**	1.06 ± 0.16	1.07 ± 0.16			
	PLx	0.932 ± 0.241	0.775 ± 0.120			
Mn-SOD	**Placebo**	1.03 ± 0.13	1.16 ± 0.33			
	PLx	1.09 ± 0.19	0.970 ± 0.190			
HO-1	**Placebo**	1.09 ± 0.22	0.557 ± 0.115			
	PLx	0.845 ± 0.150	0.814 ± 0.143			
UCP-3	**Placebo**	1.04 ± 0.15	0.874 ± 0.100			
	PLx	1.60 ±0.67	0.816 ± 0.109			
Bcl-2	**Placebo**	1.08 ± 0.21	0.762 ± 0.140			
	PLx	0.971 ± 0.088	0.933 ± 0.150			

Statistical analysis: Two-way ANOVA. (E) significant effect of exercise; (S) significant effect of supplementation. (ExS) significant interaction between the two ANOVA factors. No significant differences, $p<0.05$.

maintained the basal values after exercise. This different behavior in the antioxidant enzyme changes in erythrocytes produced significantly higher activities of glutathione peroxidase (about 20%) and reductase (about 19%) after exercise in the group supplemented with PLx than in the placebo one. In a similar way, the superoxide dismutase activity of lymphocytes increased about 51% in the group supplemented with PLx and only about 32% in the placebo group after exercise, although the differences in lymphocyte SOD activity after exercise were not statistical significant between both groups. In order to elucidate the origin of the lymphocyte changes in the superoxide dismutase activity induced by exercise we determined the gene expression of this enzyme and others antioxidant genes in lymphocytes (Table 6). No effects of exercise or supplementation were observed in the gene expression of the antioxidant enzymes superoxide dismutase, glutathione peroxidase and catalase. Other proteins with antioxidant function such as UCP3 and Bcl-2 also maintained the same basal levels of gene expression after exercise in both the placebo and the PLx supplemented groups. The gene expression of heme oxygenase, an indicator of oxidative stress, was not influenced by supplementation neither by exercise.

The effects of supplementation and exercise on the oxidative damage of lymphocytes are shown in Table 7. No effects of exercise nor supplementation were observed in the lymphocyte levels of MDA (indicator of lipid peroxidation), carbonyl index (indicator of protein modification), percentage of DNA in tail (indicator of the amount of DNA damaged in an individual lymphocyte) and in the tail moment (indicator of the degree of fragmentation of DNA in an individual lymphocyte). However, exercise significantly increased the percentage of high-damaged lymphocytes about 2.8 times in the placebo group and 1.5 times in the PLx-supplemented group. The percentage of low-damaged lymphocytes also increased after exercise but in a lower extension than the percentage of high-damaged cells. In correspondence, the percentage of intact cells decreased after exercise.

The main feature of this experience was the effect on the sexual hormone levels induced by the supplementation of the diet with PLx (Table 8). PLx significantly decreased the levels of 17-β-estradiol and both the total and free testosterone and significantly increased the levels of the sexual hormone binding protein (SHBG) in basal and after exercise conditions. 17-β-stradiol levels in the PLx supplemented group were about 48% and 67% lower than placebo in basal and after exercise conditions, respectively. In the same way, the testosterone and free testosterone levels in the PLx supplemented group were about 25% and 48% lower than placebo in basal conditions and 15% and 38% lower than placebo after exercise. In contrast, the sexual hormone binding protein was about 46% higher in PLx-supplemented group than placebo, both in basal and after exercise conditions. Exercise significantly decreased the levels of testosterone, but unaffected the levels of 17-β-stradiol, free testosterone and

Table 7. Effects of a swimming session and a supplementation with *Lippia citriodora* extract (PLx) on lymphocyte oxidative damage.

		Before	After	ANOVA E	S	ExS
MDA (μM)	**Placebo**	1.05 ± 0.11	0.677 ± 0.024			
	PLx	0.987 ± 0.067	1.08 ± 0.35			
Carbonyl index (%)	**Placebo**	100 ± 10	129 ± 13			
	PLx	136 ± 17	145 ± 17			
% DNA in tail	**Placebo**	7.70 ± 0.20	7.95 ± 0.18			
	PLx	7.83 ± 0.13	8.30 ± 0.19			
Tail moment	**Placebo**	0.300 ± 0.010	0.334 ± 0.013			
	PLx	0.290 ± 0.020	0.315 ± 0.020			
Undamaged cells (%)	**Placebo**	87.4 ± 1.4	82.2 ± 1.3*			
	PLx	89.0 ± 1.2	83.7 ± 2.4*	x		
Low-damaged cells (%)	**Placebo**	11.0 ± 0.9	13.4 ± 1.2			
	PLx	8.25 ± 0.63	12.0 ± 1.5*	x		
High-damaged cells (%)	**Placebo**	1.60 ± 0.68	4.40 ± 0.40*			
	PLx	2.75 ± 0.63	4.25 ± 1.11	x		

Statistical analysis: Two-way ANOVA. (E) significant effect of exercise; (S) significant effect of supplementation. (ExS) significant interaction between the two ANOVA factors. (*) significant differences between before and after the swimming session, $p<0.05$.

SHBG. The progesterone levels were maintained in the basal levels after exercise and no differences were observed attributable to the diet supplementation.

Table 8. Effects of a swimming session and a supplementation with *Lippia citriodora* extract (PLx) on sex hormone plasmatic levels.

		Before	After	ANOVA E	S	ExS
Progesterone (ng/ml)	**Placebo**	0.975 ± 0.102	0.785 ± 0.152			
	PLx	1.12 ± 0.25	1.11 ± 0.18			
17-β-estradiol (pg/ml)	**Placebo**	129 ± 36	144 ± 43			
	PLx	66.2 ± 6.7	47.7 ± 15.0		x	
SHBG (nM)	**Placebo**	46.4 ± 10.6	45.5 ± 9.9			
	PLx	68.6 ± 6.4	65.4 ± 6.8		x	
Testosterone (pg/ml)	**Placebo**	708 ± 53	476 ± 34 *			
	PLx	517 ± 86 #	405 ± 36	x	x	
Free testosterone (pM)	**Placebo**	36.6 ± 5.7	25.0 ± 3.0			
	PLx	19.0 ± 2.7 #	15.5 ± 1.2		x	

Statistical analysis: Two-way ANOVA. (E) significant effect of exercise; (S) significant effect of supplementation. (ExS) significant interaction between the two ANOVA factors. (*) significant differences between before and after the swimming session. (#) significant differences between placebo and PLx supplementation, $p<0.05$.

Discussion

The duration and the intensity of exercise influence the induction of oxidative damage [53]. The duration and intensity of the exercise test applied in the present study induced a low immune response as shown by the low increase in the circulating neutrophil counts. Neutrophils only increased about 32% after 30 minutes of swimming at 80% of the maximal register; whereas in other longer and more intense exercise tests the increase in the neutrophil counts is much higher [41, 53, 54]. The increase in the circulating neutrophils observed after the swimming session is probably associated to an immune response to exercise that include neutrophil priming to oxidative burst, as seen after a football match or a cycling stage [41, 53]. The diet supplementation with moderate levels of antioxidants such as vitamins E and C or ubiquinone reduces plasma oxidative damage induced by intense exercise as a half-marathon [13, 55] or a football match [54], but without blocking the cellular adaptation to exercise [13, 54]. However, the diet supplementation with high doses of vitamin C, in as much as 1 g/d for one week, reduces neutrophil response to hypoxia/reoxyge-nation associated to apnea diving [15]. Whereas apnea diving induces oxidative stress and initiates a neutrophil reaction that resemble the acute-phase immune response, the supplementation with 1g/day of vitamin C reduces iNOS levels and NO production by neutrophil [15]. Then, it is evident that the antioxidant intake influences the oxidative damage and the cellular adaptations against the oxidative stress induced by intense exercise. The swimmers participating in this study intook high levels of antioxidants during the nutritional intervention, coming both from food and the almond based beverage used as the placebo and the vehicle to intake the PLx extract. The antioxidant vitamin intakes (vitamins C and E) were at the same level in both the placebo and the PLx supplemented groups, and were between 2.5 and 5 times higher than the RDA for general people [56]. We have previously observed that the oxidative damage induced by intense exercise is well balanced by the availability of antioxidant nutrients [13, 54]. Actually, no effects of swimming for 30 min at 80% of maximal register are observed on the markers of oxidative damage in lymphocytes of woman swimmers fed with doses of vitamins C and E five times higher than their RDA. In spite of this apparent lack of oxidative damage induced by exercise in lymphocytes, the number of lymphocytes with damaged DNA was about 3 times higher after the swimming session than in basal conditions, although only about 4% of cells had DNA damage after exercise. The additional supplementation with PLx did not affect the number of lymphocytes with damaged DNA after swimming.

The supplementation with PLx slightly reduced the number of erythrocytes and leukocytes. The reduction in the number of erythrocytes is parallel to a significant reduction of hematocrit and blood hemoglobin, while the significant reduction of leukocytes is reflected in a non significant reduction in lymphocytes, monocytes eosinophils and basophils. This picture is present both in basal conditions and after exercise. The supplementation with antioxidant vitamins decreases the number of circulating lymphocytes, but maintaining the lymphocyte count in the normal range [57]. Although some studies revealed that supplementation with vitamin E can increase lymphocyte proliferation [58], in a previous experiment in which amateur runners consumed the same beverage here used as the placebo we also detected a decrease in the number of circulating lymphocytes [57]. Supplementation with either 1 g/d vitamin C and 500 mg/d vitamin E [59] or 1 g/d of vitamin C alone [60] has also been shown to induce lymphopenia. In the present study, the PLx-supplemented group intook higher levels of

antioxidants than the placebo because of the presence of PLX extract. We also find a certain degree of leukopenia in this PLX supplemented when compared to the placebo group. The mechanisms by which the supplementation with antioxidant nutrients induces lymphopenia are not clear, although an increase in the levels of corticosteroids in response to increased ascorbate levels could be involved [61, 62].

In spite of the lack of oxidative damage, the exercise increased the activity of superoxide dismutase in lymphocytes. This enzyme is one of the first enzymes activated against oxidative stress induced by exercise [63, 64]. The activation is observed both in the placebo and in the PLx supplemented group, although the increase is higher in the PLx supplemented group. This pattern is not attributable to a change in the gene expression of Mn-SOD. Therefore, the increased activity of superoxide dismutase after exercise could be attributed to the activation of preexistent superoxide dismutase protein in lymphocytes. In fact it has been pointed out that exercise induces the activation of some enzymes in erythrocytes as a result of post translational regulation [65, 66]. The lack of oxidative damage after exercise is also in accordance with a lack of effects on the antioxidant gene expression in lymphocytes, because there is a certain parallelism between the induction of oxidative damage and the induction of the antioxidant enzymes in lymphocytes [12].

The activities of some key antioxidant enzymes increased in erythrocytes as a result of the interaction between exercise and the PLx supplementation. This change in the antioxidant activity has to be attributed to actions on the enzymatic protein present in the erythrocyte, because erythrocytes are unable to synthesize proteins [67]. Intense exercise such as a duathlon race or a mountain cycling stage induce changes in the erythrocyte activity of glutathione peroxidase [65, 68]. As shown in the present study, the diet supplementation with PLx enhances the activation of glutathione dependent enzymes in erythrocytes induced by a short and intense exercise.

The main contribution of this study is the demonstration that the supplementation of the diet with PLx alters the sexual hormone circulating levels in basal conditions. An action as agonist/antagonist of steroid hormones has been attributed to verbascoside and martynoside, the main compounds presents in the Plx extract [28, 29]. We here evidence that the intake of 2g/day of Plx extract for 26 days (about 200mg/d verbascoside) significantly decreases the plasma levels of estradiol and testosterone and increases the level of the sexual hormone binding protein, probably as a result of the agonist effect between verbascoside and estradiol. The synthesis of estradiol is regulated by negative feedback by the same estradiol, which stimulates the secretion by the hypophysis of the hormone which inhibits the ovaric production of estradiol. By acting as an estrogenic agonist, verbascoside could act inhibiting estradiol synthesis. In fact these phytoestrogens may act as estrogen agonists/antagonists via the known estrogen receptor alpha (ERα) and beta (ERβ) subtypes [27].

In summary, the diet supplementation with phytoestrogens from *Lippia citriodora* such as verbascoside decrease the number of circulating erythrocytes and leukocytes and enhances the glutathione-dependent enzyme activities in erythrocytes and the superoxide dismutase activity in lymphocytes in response to a short and intense exercise. The diet supplementation with verbascoside from *Lippia citriodora* also decreases the plasma steroid hormone levels, pointing out a possible agonist effect between estradiol and these phytoestrogens.

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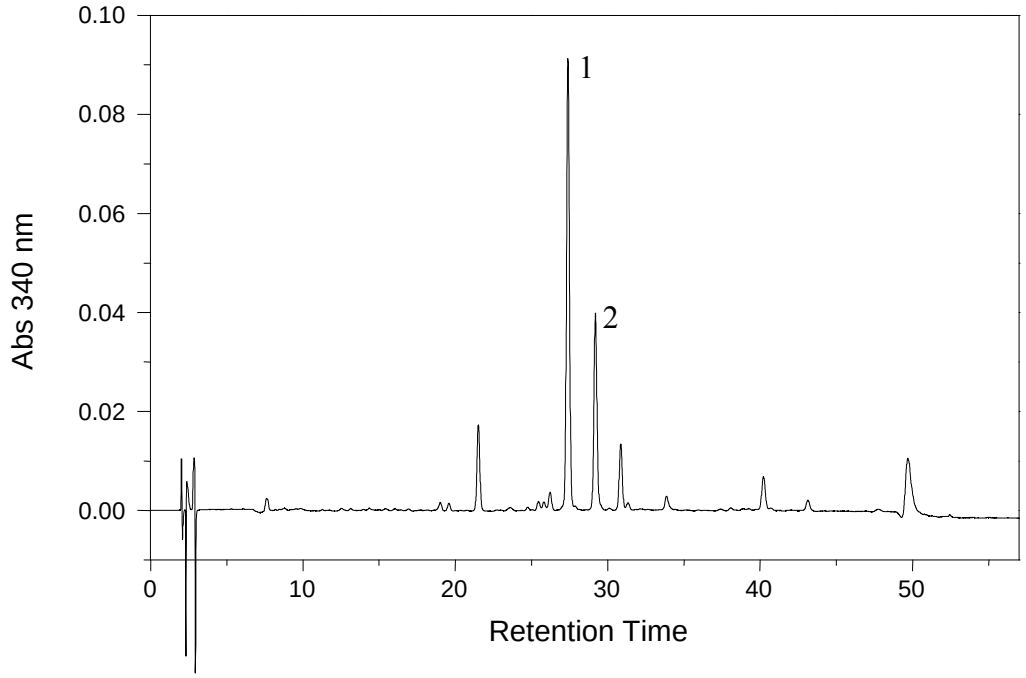
FIGURES

Figure 1. High-performance liquid chromatography profile of the *Lippia citriodora* extract at 340 nm.



(1= verbascoside; 2= isoverbascoside).

Manuscript XV

The double edge of reactive oxygen species as damaging and signaling molecules in HL60 cell culture.

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The double edge of reactive oxygen species as damaging and signaling molecules in HL60 cell culture

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Abstract

The reactive oxygen species (ROS) are a double-edged sword because they serve as key signal molecules in physiological processes but they also have a role in pathological processes. Our aim was to evidence the double effects of ROS by establishing the conditions in which the ROS produce pathological or hormetic effects on HL60 cells, and evidence the induction effects of hydrogen peroxide on the expression of antioxidant genes and transcription cofactors. HL60 cells were treated with either single bouts of 1, 10 and 100 μM H_2O_2 or a sustained H_2O_2 production of 0.1, 1 and 10 nM H_2O_2/s. The exposure to 10 and 100 μM activated catalase, glutathione peroxidase and glutathione reductase through post-transcriptional mechanisms and induced oxidative modification of proteins. When cells where exposed to sustained H_2O_2 production, a clear dose-response effect was detected in the activity of the antioxidant enzymes catalase, glutathione peroxidase and Mn-SOD, with higher concentrations of H_2O_2 inducing higher enzyme activities. Catalase, HO-1, UCP-3, iNOS and PGC-1α expressions were activated after sustained exposure to 1 and 10 nM/s. Although the antioxidant defenses were activated, oxidative damage appeared in DNA and proteins in cells treated with 1 and 10 nM/s. In conclusion, HL60 cells respond to exposure to sustained levels of H_2O_2 in a dose-response manner to the H_2O_2 concentration by activating the expression and activity of the antioxidant machinery, although the activation of the antioxidant defenses is not enough to avoid the apparition of oxidative damage. Of the two designs proposed the continuous exposure seems to be more appropriate to study the antioxidant response of HL60 cells to H_2O_2. The experimental design using glucose/glucose oxidase to produce hydrogen peroxide in a sustained manner allows reproducing *in vitro* hormetic effects of ROS.

Introduction

The reactive oxygen species (ROS) are a double-edged sword because they serve as key signal molecules in physiological processes [1-3] but they also have a role in pathological processes such as cachexia [4], atherosclerosis [5], cancer [6] and neurodegenerative diseases such as Alzheimer and Parkinson diseases [7]. The most accepted theory of aging also incorporates the damaging effects of ROS, that are considered to be unavoidable by-products of aerobic metabolism [8, 9]. As in other cases, it is the dosage the main variable that influences the side of the double-edge which ROS represent in each moment. The typical reaction to ROS can be described by a bell-shaped curve: low concentrations have a stimulating effect (signalling, receptor stimulation, enzymatic stimulation), while a massive level of ROS inhibits enzyme activity and causes apoptosis or necrosis. The hormesis theory states that biological systems respond with a bell-shaped curve to exposure to chemicals, toxins, and radiation, and this theory has been expanded to reactive oxygen species [10-12]. It has been clearly shown that a single bout of exercise above a certain intensity or duration results in increased production of ROS and causes oxidative damage to lipids, proteins, and DNA [13-15]. On the other hand, it is also well established that regular exercise is a preventive measure against oxidative stress-related diseases including cardiovascular diseases, stroke, and certain cancers [16-18]. In parallel, it is also pointed out that the diet supplementation with some antioxidant nutrients enhances the induction of the endogenous antioxidant system by exercise [19] and it has been also described that the dietary supplementation with flavonoids reduces the incidence of myocardial infarction [20], plasma LDL cholesterol concentration [21], and lipid peroxidation [22].

The HL60 cell line was established from a patient with acute promyelocytic leukaemia and consists predominantly of promyelocytes [23]. Although it is an undifferentiated cell line it can differentiate into either monocytes/macrophages or granulocytes/neutrophils after certain stimulus such

as PMA and retinoic acid, respectively [24, 25]. Oxidative stress associated to exposure to H_2O_2 has been studied in HL60. The exposure of cells to 40-50 μM H_2O_2 has been shown to induce oxidative damage in DNA [26], but also activate the expression of genes implicated in NF-κB activation, transcription and DNA methylation along with cytokines and cytokine receptors [27].

The rate of ROS production and the doses at which the cells are exposed to are crucial to express the adaptative or pathological responses of the cell. Our aim was to evidence the double effects of ROS by establishing the conditions in which the ROS produce pathological or hormetic effects on HL60 cells, and evidence the induction effects of hydrogen peroxide on the expression of antioxidant genes and transcription cofactors. Two situations have been assayed for this purpose. The first situation consists on the exposure of HL60 cells to a bolus of high hydrogen peroxide concentration. In this situation the antioxidant defences of cells are overwhelmed and cell death is induced. The second situation consists on the exposure of HL60 cells to a continuous and low production of hydrogen peroxide. In this situation, the stationary state with an excess of hydrogen peroxide leads to a new redox and more oxidized equilibrium in which the antioxidant defences are activated although the cellular components are more oxidized.

Materials and methods

Cell culture

HL60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin, 0.1 ng/ml streptomycin and 2mM L-glutamine, in a humidified atmosphere containing 5% CO2 at 37ºC. The cells were cultured into 250 ml tissue culture flasks at the initial concentration of 2 x 10^5 cells/ml.

Cell treatments and experimental design

All the treatments were performed on 6-well plates containing 6 x 10^5 cells/ml. Two experimental designs were performed. In the first experience cells were treated with single bouts of H_2O_2. The final concentrations of H_2O_2 used were 1, 10 and 100 μM. Two hours after the addition of H_2O_2 cells were harvested and washed twice in PBS. In the second experience, cells were incubated for one hour in the presence of glucose and glucose oxidase (GOX) with a controlled GOX activity to generate a rate of hydrogen peroxide production of 0.1, 1 and 10 nM H_2O_2/s [28]. GOX activity was previously determined with 5 mM glucose as substrate with the method described below. One hour after the addition of the treatment cells were washed twice in PBS and incubated for one more hour in culture medium. Total protein concentrations were measured by the method of Bradford [29].

H_2O_2 concentration and glucose oxidase activity determination

The change in concentration of H_2O_2 in the culture medium both in the presence and absence of cells after the addition of 1, 10 and 100 μM H_2O_2 was monitored colorimetrically using horseradish peroxidase and tetramethylbenzidine (TMB). The H_2O_2 concentration was calculated with a standard curve of known concentration. H_2O_2 production by glucose oxidase was also determined with this method using 5 mM glucose as substrate.

Cell viability

Cell viability was measured using the MTT method. Briefly, cells were treated with all treatments (single bouts and continuous H_2O_2 production) for 1 hour. After a washing procedure with PBS (to remove all enzymes), MTT was added to each well (0.5 mg/ml) and cells were incubated for 4 h at 37ºC. The plates were then centrifuged and supernatant was discarded. Tetrazolium crystals were resuspended in DMSO and the absorbance was measured at 570/620 nm.

Enzymatic determinations

Catalase (CAT) activity was measured by the spectrophotometric method of Aebi using H_2O_2 as substrate [30]. Glutathione reductase (GRd) activity was measured by the Goldberg and Spooner spectrophotometric method using oxidized glutathione as the substrate [31]. Glutathione peroxidase (GPx) activity was measured using the spectrophotometric method of Flohé and Gunzler [32]. This assay required H_2O_2 and NADPH as substrates and glutathione reductase as enzyme indicator. Superoxide dismutase (SOD) activity was measured by the method of McCord & Fridovich [33]. The xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion produced the reduction of cytochrome *c*, which was monitored at 550 nm. The superoxide dismutase in the sample removed the superoxide anion and produced an inhibition of the cytochrome *c*

Table 1. Primers and conditions used in Real Time PCRs.

Gene	Primers	Conditions
18S	Fw: 5'-ATG TGA AGT CAC TGT GCC AG-3' Rv: 5'-GTG TAA TCC GTC TCC ACA GA-3'	95ºC,10s; 60ºC, 7s; 72ºC, 12s
Catalase	Fw: 5'-TTT GGC TAC TTT GAG GTC AC-3' Rv: 5'-TCC CCA TTT GCA TTA ACC AG-3'	95ºC,10 s; 60ºC, 10s; 72ºC, 15s
GPx	Fw: 5'-TTC CCG TGC AAC CAG TTT G-3' Rv: 5'-TTC ACC TCG CAC TTC TCG AA-3'	94ºC, 1s; 60ºC, 7s; 72ºC, 5s
Bcl-2	Fw: 5'-CTG GTG GGA GCT TGC ATC AC-3' Rv: 5'-ACA GCC TGC AGC TTT GTT TC-3'	95°C, 5s; 65ºC, 5s; 72ºC, 5s
SIRT3	Fw: 5'-GAG CTT CTG GGC TGG ACA GA-3' Rv: 5'-TGG GAT GTG GAT GTC TCC TAT G-3'	95°C, 10s; 65ºC, 5s ; 72ºC, 7s
PGC-1α	Fw: 5'-TCA GTC CTC ACT GGT GGA CA-3' Rv: 5'-TGC TTC GTC GTC AAA AAC AG-3'	95°C, 10s; 60ºC, 10s; 72ºC, 15s
UCP-3	Fw: 5'- CGTGGTGATGTTCATAACCTATG-3' Rv: 5'- CGGTGATTCCCGTAACATCTG-3'	95°C, 5s; 60ºC, 7s; 72ºC, 10s
Mn-SOD	Fw: 5'-GAG AAG TAC CAG GAG GCG TTG-3' Rv: 5'- CAA GCC AAC CCC AAC CTG AGC-3'	95ºC, 30s; 64ºC, 60s
HO-1	Fw: 5'-CCA GCG GGC CAG CAA CAA AGT GC-3' Rv: 5'-AAG CCT TCA GTG CCC ACG GTA AGG-3'	95ºC, 0s; 60ºC, 5s; 72ºC, 10s
iNOS	Fw: 5'-TCTGCAGACAGTGCGTTACT-3' Rv: 5'-ATGCACAGCTGAGCATTCCA-3'	95ºC, 10s; 60ºC, 10s; 72ºC, 15s

reduction. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37ºC.

mRNA gene expression

mRNA expressions were determined by real time RT-PCR with 18S ribosomal as reference gene. For this purpose, mRNA was isolated by extraction with Tripure Isolation Reagent (Roche). cDNA was synthesized from 1 µg total RNA using reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. The primers used are shown in Table 1. For all PCRs there was one cycle at 95ºC for 10 min, followed by 40 cycles at the conditions shown in Table 1.

The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Basal mRNA levels at the beginning of the stage were arbitrarily referred to as 1. The expression of the target gene was normalized with respect to ribosomal 18S.

Hydrogen peroxide production

H_2O_2 production was measured using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as indicator. A stock solution of DCFH-DA (1 mg/ml) in ethanol and PMA (1mg/ml) in DMSO were prepared, and stored at -20°C until analysis. DCFH-DA (30 µg/ml) in PBS was added to a 96-well microplate containing 50 µl cell suspension (containing about 6 x 10^5 cells) The fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37ºC for 1 hour in FLx800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc.).

Nitrite determination

Nitrite levels were determined in the culture medium by the acidic Griess reaction using a spectrophotometric method. Cell samples were centrifuged 10 min at 900xg at 4ºC. Supernatants were collected and deproteinized with acetone and kept overnight at –20ºC. Samples were centrifuged for 10 min at 15000xg at 4ºC, and supernatants were recovered. A 96-well plate was loaded with the samples or standard nitrite solutions (100 µl) in duplicate. 50 µl sulfanilamide (2% w/v) in 5% HCl was added to each well, and 50 µl *N*-(1-napthyl)-ethylenediamine (0.1% w/v) in water was then added. The absorbance was measured at 540 nm following an incubation of 30 min. The nitrite concentration was calculated with a standard curve of known concentration.

Protein carbonyl determination

Protein carbonyl derivatives were determined by an immunological method using the OxyBlot™ Protein Oxidation Detection Kit (Chemicon International) following manufacturer's details. Briefly, 10 μg of protein were incubated in the presence of 2,4-dinitrophenylhydrazine (DNPH). Once derivatized, samples were transferred to a nitrocellulose membrane by the method of dot blot. The membrane was then incubated with primary antibody, specific to the DNP moiety of the proteins. This step was followed by incubation with a horseradish peroxidase-antibody (goat anti-rabbit IgG) conjugate directed against the primary antibody. The membranes were then treated with luminol, which is converted to a light-emitting form at wavelength 428 nm by the antigen/primary antibody/secondary antibody/peroxidase complex. The light was visualized and detected by short exposure to a Molecular Imager Chemidoc XRS (Bio-Rad Laboratories). Image analysis was performed using Quantity One-1D analysis software (Bio-Rad Laboratories).

Comet assay

Assessment of DNA damage was carried out using the comet assay method. Briefly, slides were prepared by adding 20 ml purified lymphocytes, mixed with 80 ml 0.6% low-melting-point agarose. In order to release the DNA, cells were lysed by immersing slides in lysing solution (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mM Tris, 1% Triton X-100 and 10% DMSO added freshly, pH 10) at 4ºC. DNA was denatured by placing slides in alkaline bath in the electrophoresis tank for 40 min to allow for the unwinding of the DNA and expression of alkali-labile damage. Electrophoresis was carried out with an electric current of 250 mV applied for 30 min. Tris buffer pH 7.5 was added onto the slides to neutralize excess alkali. Finally, DNA was stained by adding 80 ml ethidium bromide (20 mg/ml) to each slide.

Comet measurements were made by image analysis using a fluorescence microscope and the Comet software (TriTek CometScore™, version 1.5). Images of 100 randomly selected nuclei were analysed for each sample.

Statistical analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 13.0 for Windows). Results are expressed as mean ± s.e.m. and $P<0.05$ was considered statistically significant. The statistical significance of the data was assessed by a one-way ANOVA.

Results

Two experimental designs were performed to study the endogenous antioxidant response of HL60 cells to both an acute and a chronic exposure to H_2O_2. To study the effects of an acute bout of hydrogen peroxide we tested three different initial concentrations of H_2O_2: 1, 10 and 100 μM. H_2O_2 disappearance in the culture medium was followed for 1 hour, and the results are shown in Figure 1. H_2O_2 disappearance in the presence of cells followed a first order kinetic when H_2O_2 was added at an initial concentration of 10 and 100 μM. 15 minutes after the beginning of the treatment the levels of H_2O_2 in the medium had been reduced to a half in both of the treatments, and after 60 minutes the exogenously added H_2O_2 had been almost completely depleted. The kinetic constants (k) for the disappearance of H_2O_2 were 15.8 and 12.2 $k/10^9$ cells after the addition of 10 and 100 μM H_2O_2, respectively.

When cells were exposed to an acute bout of H_2O_2, the response was influenced by the dosage of H_2O_2 provided. The initial concentration of 10 μM H_2O_2 was the only one to induce the activity of all the antioxidant enzymes studied (Table 2). The exposure to 1 μM H_2O_2 shown little cell response, only activating catalase activity. The exposure to 100 μM induced the activity of catalase, glutathione peroxidase and glutathione reductase, but not superoxide dismutase. These changes in enzyme activities were not due to transcriptional changes because none of the genes encoding these enzymes shown increased expression. Catalase, glutathione peroxidase, Mn-SOD and heme oxygenase 1 (HO-1) expressions were even reduced in the cells treated with 100 μM. The reduction in the expression of these genes was accompanied by the reduction in the expression of the antiapoptotic protein Bcl-2, in accordance with the higher cell death shown by this group. On the other hand, the endogenous H_2O_2 production of HL60 cells was decreased when the cells were exposed to 100 μM (Table 3). We then studied if the activation of the antioxidant defenses was enough to avoid oxidative damage. Oxidation of proteins appeared after the addition of the same H_2O_2 concentrations that induced the antioxidant enzymes, thus suggesting that the activation of the antioxidant defenses runs parallel to the apparition of oxidative damage (Table 3). Cell viability after

Table 2. Antioxidant enzyme activities and gene expression in HL60 treated with growing levels of H_2O_2.

	Control	1 μM	10 μM	100 μM
Enzyme activities				
Catalase (k/mg)	111 ± 3	144 ± 6*	184 ± 17*#	220 ± 9*# §
GPx (nkat/mg)	337 ± 11	331 ± 5	410 ± 21*#	415 ± 4*#
GRed (nkat/mg)	449 ± 36	490 ± 17	665 ± 43 *#	546 ± 23* §
MnSOD (pkat/mg)	125 ± 12	136 ± 5	164 ± 10*#	140 ± 9 §
Gene expression				
Catalase	1.01 ± 0.11	1.28 ± 0.19	0.626 ± 0.075#	0.577 ± 0.085*#
GPx	1.00 ± 0.02	1.00 ± 0.17	1.06 ± 0.11	0.480 ± 0.082*#§
MnSOD	1.05 ± 0.20	1.00 ± 0.18	0.973 ± 0.117	0.491 ± 0.070
HO-1	1.01 ± 0.12	0.567 ± 0.015*	0.410 ± 0.065*	0.423 ± 0.044*
UCP3	1.08 ± 0.29	1.22 ± 0.15	1.27 ± 0.37	1.12 ± 0.45
Bcl-2	1.00 ± 0.01	1.02 ± 0.12	0.905 ± 0.013	0.523 ± 0.022*#§
PGC-1α	1.10 ± 0.29	1.07 ± 0.30	1.20 ± 0.53	0.549 ± 0.285

The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels of the control group were arbitrarily referred to as 1. Statistical analysis: One-way ANOVA. (*) Significant differences vs Control. (#) Significant differences vs 0.1 nM. (§) Significant differences vs 1 nM, $p<0.05$

the H_2O_2 treatments is also shown in Table 3. The treatment with 1 and 10 μM H_2O_2 did not influence cell viability. The treatment with 100 μM H_2O_2, however, induced cell death and reduced the viability to about the 80% of control cells.

Figure 2 shows H_2O_2 production by the glucose/GOX system in the absence of cells. All treatments maintained a constant H_2O_2 production for the 60 minutes of the treatment. Three sustained productions of H_2O_2 were used: 0.1, 1 and 10 nM/s. When cells were exposed to a sustained production of H_2O_2 for 1 hour instead of a single bout the response was clearly different. One hour after returning to culture medium without hydrogen peroxide a clear dose-response effect was detected in the activity of the antioxidant enzymes catalase, glutathione peroxidase and Mn-SOD, with higher concentrations of H_2O_2 inducing higher enzyme activities (Table 4). 0.1 nM/s H_2O_2 only induced the activity of Mn-SOD, however exposure to 1 and 10 nM/s H_2O_2 induced the activity of catalase, GPx and Mn-SOD, and the higher activities were always found in the cells exposed to 10 nM/s H_2O_2. These changes in enzyme activity could be, at least in part, consequence of increased gene expression, as catalase mRNA levels shown an identical response to enzyme activity. Other antioxidant defenses such as HO-1 and UCP-3 and the expression of iNOS were also activated at the transcriptional level when the increased productions of H_2O_2 attained 10nM/s, as shown in Table 4. The activation of all these genes was accompanied by the activation of the coactivator PGC-1α. Table 5 shows the production of both ROS and RNS, together with oxidative damage and cell viability. Both endogenous H_2O_2 production and the extracellular nitrite levels showed a dose-response effect to extracellular H_2O_2 exposure. Cells treated with 1 nM/s H_2O_2 produced higher levels of H_2O_2 and nitrite than controls, and cells treated with 10 nM/s H_2O_2 shown higher nitrite levels and H_2O_2 production capability than any other group. The increase in nitrite production was subsequent to the activation of iNOS expression, especially in the 10 nM/s group. The activation of

Table 3. ROS production, oxidative damage and cell viability in HL60 treated with growing levels of H_2O_2.

	Control	1 μM	10 μM	100 μM
H_2O_2 (RFU/min/mg)	100 ± 14	83 ± 14	102 ± 20	54.1 ± 5.1* §
Carbonyl index (%)	100 ± 21	143 ± 17	181 ± 17 *	223 ± 45 *#
Cell viability (%)	100 ± 1.0	96.0 ± 2.7	98.6 ± 1.1	82.8 ± 0.6 *#§

Statistical analysis: One-way ANOVA. (*) Significant differences vs Control. (#) Significant differences vs 0.1 nM. (§) Significant differences vs 1 nM, $p<0.05$

Table 4. Antioxidant enzyme activities and gene expression in HL60 treated with growing sustained levels of H_2O_2.

	Control	0.1 nM/s	1 nM/s	10 nM/s
Enzyme activities				
Catalase (k/mg)	107 ± 9	114 ± 5	136 ± 7*	157 ± 5*#
GPx (nkat/mg)	172 ± 9	219 ± 24	325 ± 38*	385 ± 30*#
GRed (nkat/mg)	423 ± 5	422 ± 8	430 ± 7	418 ± 3
MnSOD (pkat/mg)	145 ± 5	187 ± 4*	198 ± 6*	204 ± 4*#
Gene expression				
Catalase	1.00 ± 0.21	2.51 ± 0.54	3.44 ± 0.55*	5.05 ± 0.71*#
HO-1	1.00 ± 0.15	2.54 ± 0.23	4.78 ± 0.46*#	7.45 ± 0.78*# §
UCP3	1.00 ± 0.18	2.03 ± 0.17	3.42 ± 0.44	12.9 ± 2.0*# §
iNOS	1.00 ± 0.10	1.37 ± 0.07	1.50 ± 0.08	1.95 ± 0.31 *#
PGC-1α	1.00 ± 0.17	3.30 ± 0.57	9.72 ± 1.53*	18.0 ± 3.8*# §

The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels of the control group were arbitrarily referred to as 1. Statistical analysis: One-way ANOVA. (*) Significant differences vs Control. (#) Significant differences vs 0.1 nM/s, (§) Significant differences vs 1 nM/s,$p<0.05$

cell defenses was not enough to avoid the early appearance of oxidative damage as shown by the higher carbonyl index in all treated groups when compared to controls. Not only proteins but also DNA got damaged, as shown by the increased %DNA in tail in all the treated groups. The increased oxidative damage markers were however not accompanied by cell death, as cell viability was maintained through the treatments.

Discussion

The exposure of cells to reactive oxygen species (ROS) has been widely described to induce the appearance of oxidative damage and oxidative stress [13-15]. In many physiological or pathological processes, cells are exposed to increased levels of ROS [4, 5, 7]. However, when produced at low levels, ROS can act as signaling molecules which can induce the activation of antioxidant defenses [1-3]. In our study we show that HL60 cells respond differentially to H_2O_2 exposure depending on the levels and persistence of the exposure.

After the addition of a single bout of H_2O_2, the kinetics of its disappearance followed a first order behaviour. The H_2O_2 detoxifying enzymes present in cells are catalase and glutathione peroxidase (GPx). While GPx needs a cosubstrate (glutathione) to function, catalase activity only depends on H_2O_2 availability. Therefore the presence of a first order kinetic of H_2O_2 disappearance points to catalase as the main H_2O_2 consuming enzyme in these conditions. When HL60 cells were exposed to a bolus of H_2O_2, they responded by activating their antioxidant enzyme defenses, but not all antioxidant enzymes responded equally. Catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase activities were increased when cells were treated with 10 μM H_2O_2 but not with 1 μM. Catalase, glutathione peroxidase and glutathione reductase were also activated in the cells treated with 100 μM H_2O_2, but only catalase responded fully in a

Table 5. ROS production, oxidative damage and cell viability in HL60 treated with growing sustained levels of H_2O_2.

	Control	0.1 nM/s	1 nM/s	10 nM/s
H_2O_2 (RFU/min/mg)	143 ± 3	154 ± 2	163 ± 3*	233 ± 11*# §
Nitrite (nmol/ml)	4.02 ± 0.3	12.3 ± 1.0	41.4 ± 7.8*#	86.5 ± 8.7*# §
Carbonyl index (%)	100 ± 5	212 ± 19*	261 ± 28*	297 ± 45*
%DNA in tail	7.61 ± 0.57	9.43 ± 0.77	9.89 ± 0.93*	10.7 ± 0.93*
Tail moment	0.317 ± 0.029	0.372 ± 0.046	0.398 ± 0.036	0.485 ± 0.053*
Cell viability (%)	100 ± 1.0	98.2 ± 2.3	99.4 ± 2.4	98.8 ± 1.7

Statistical analysis: One-way ANOVA. (*) Significant differences vs Control. (#) Significant differences vs 0.1 nM. (§) Significant differences vs 1 nM, $p<0.05$

dose-response manner, with the higher activities shown in the cells treated with 100 μM. Glutathione reductase and superoxide dismutase activities were in contrast lower in the 100 μM group when compared to the 10 μM. The activation of the antioxidant enzymes in the cells treated with 10 μM H_2O_2 was not due to increased gene expression, as shown by the maintained expression patterns of all these genes. Furthermore, in the cells treated with 100 μM we even observed a reduction in the expression of catalase and glutathione peroxidase, in contrast with the increased enzyme activities. Then, the activation of antioxidant enzymes in this cases are a result of post-transcriptional modifications. We have previously evidenced that some enzymes in erythrocytes increase their activity *in vivo* after intense exercise, due to direct effects on the enzymatic protein that produce an increase in the specific activity of this enzymes [34, 35]. A bolus of 10 μM H_2O_2 is enough to increase the activity of these antioxidant enzymes, thus reinforcing the possible activation effects of ROS on the activity of these proteins. However, a different behaviour is observed depending on the antioxidant enzyme. Catalase increases its activity as the concentration of hydrogen peroxide increases; GPx also increases its activity, but attaining a plateau between 10 and 100 μM H_2O_2; GRd and SOD present a bell-shaped curve with the higher activity shown at 10 μM H_2O_2. Although the antioxidant defenses were activated in the cells treated with 10 and 100 μM H_2O_2, this activation was not enough to avoid the apparition of oxidative damage in proteins. The expression of the antiapoptotic protein Bcl-2 is reduced after the addition of 100 μM H_2O_2, thus indicating that an apoptotic process could have been initiated in accordance with the reduced cell viability observed with the MTT assay. Several experiences in which hydrogen peroxide induces cell death, apoptosis or oxidative damage use H_2O_2 concentrations above 100 or 200 μM ([36-40]). A concentration of 100 μM H_2O_2 is then enough to induce apoptosis in HL60, while the apoptotic process is not initiated after exposure up to 10μM H_2O_2. The results obtained with this experimental design show that the exposure of HL60 cells to an isolated dose of 10 μM H_2O_2 enhances the activity of all the antioxidant enzymes without affecting gene expression, and the activation of the enzymes seems to be effective in maintaining the cell viability, although oxidative damage appears. However, although the exposure to higher concentrations of H_2O_2 (100 μM) also enhances enzyme activities, this raise should be transient and ineffective, because the expression of all the antioxidant enzymes are reduced, oxidative damage appears and the cells get primed to apoptosis and death.

To study the effects of a sustained exposure of cells to maintained H_2O_2 levels we used a system including glucose oxidase (GOX) and glucose. Under special conditions of H_2O_2 production (e.g. at very low oxidase activities) the H_2O_2 degradation by cellular catalase or glutathione peroxidase suffice to prevent accumulation of H_2O_2 in the culture medium and no external catalase is necessary to remove the excess of H_2O_2 [28]. In the present study the GOX enzyme was diluted to adjust their H_2O_2 generation rates to a maximum of 10 nM/s. This activity is comparable to observations made in suspensions of neutrophils [41]. When cells were exposed to sustained production of H_2O_2 instead of a single bout the adaptative response was clearly different. None of the treatments induced cell death thus indicating that all concentrations used were safe for the cell and were not toxic. The exposure of cells to a sustained production of 0.1 nM H_2O_2/s was enough to enhance Mn-SOD activity but also to induce the apparition of protein oxidative damage. Both the exposures to 1 and 10 nM H_2O_2/s enhanced catalase, glutathione peroxidase and Mn-SOD activities. These enhanced activities could be a consequence of the activation of gene expression, as seen for catalase gene, but in addition they could also be attributed to changes in the specific activity, as occurs after cell exposure to a bolus of high levels of H_2O_2. As the enzyme activity was determined 1 hour after the complete removal of H_2O_2 in the medium, the activation induced by H_2O_2 on the enzymatic protein might persist even when the stimulus has disappeared. However, this pattern of response is more probably due to an induction of gene expression during the exposure to H_2O_2 that is manifest after the removal of the inductor. The fact that antioxidant gene expression is also activated when cells are exposed to sustained low levels of H_2O_2 could reflect an insurance for maintaining cell stability on a long term basis. Together with the classical antioxidant enzymes, HO-1 and UCP-3, both of them proteins with antioxidant properties [42-46], were activated when cells were exposed to the 1 and 10 nM H_2O_2/s treatments. Accumulating evidence suggests that HO-1 plays an important role in cellular protection against oxidant-mediated cell injury [42]. HO-1 is an antioxidant stress protein, a member of the family of heat shock proteins (HSP) that is mainly induced by ROS, inflammatory cytokines, and also by an acute bout of exercise [44, 47]. It seems that the protective effects of HO-1 are

related to the formation of the antioxidant molecule bilirubin resulting from heme degradation [48]. HO-1 is particularly sensitive to acute exercise being activated by the NF-kB pathway. Several authors have evidenced an increase in HO-1 after a half-marathon [49] or after a treadmill test until exhaustion [50], but not after a short run or eccentric exercise [49]. In a previous work we evidenced that HO-1 is activated depending on the degree of ROS production [51]. Our present results reinforce the possibility that HO-1 activation is dependent on the intracellular levels of ROS, because HO-1 gene expression rose significantly with every increasing H_2O_2 treatment. UCP-3 expression followed a similar pattern to HO-1. UCP-3 is a member of the mitochondrial uncoupling protein family mainly detected in skeletal muscle; it is not involved in thermogenesis [45] but has been shown to act as an antioxidant by reducing reactive oxygen species production [46]. UCP-3 expression is usually enhanced in skeletal muscle after acute bouts of physical activity, while a down-regulation is observed in response to training [52]. We have recently described that UCP-3 expression is down-regulated in lymphocytes after an intense swimming session, and that UCP-3 and Bcl-2 expressions are directly correlated [2]. PGC-1α expression pattern responded in a similar way to catalase, HO-1 and UCP-3, by enhancing gene expression in response to increasing H_2O_2 production rates. PGC-1α is a transcriptional coactivator which enhances the transcription of genes involved in mitochondrial biogenesis and uncoupling [53] and it has been recently described to co-activate the expression of antioxidant genes such as Mn-SOD and catalase, thus improving the antioxidant capacity of cells and preventing oxidative stress mediated apoptosis [54]. Exercise, which is characterized by increased ROS production, has been shown to induce the expression of PGC-1α in muscle cells [55, 56]. The expression of PGC-1a has been recently related to the expression of both HO-1 and UCP-3, thus suggesting that it could be involved in the activation of these antioxidant proteins together with the antioxidant enzymes [2, 57]. The exposure of cells to 10 nM H_2O_2/s also induced, although in a lower extension, the expression of the nitric oxide synthase iNOS and a 20-fold increase in the extracellular levels of nitrite as a marker of nitric oxide. In previous studies we have evidenced that iNOS expression is activated in lymphocytes after acute bouts of exercise only when H_2O_2 production is increased, or after exposure to hyperbaric oxygen, and this gene activation is usually accompanied by increases in cellular nitrite levels [58-60]. The expression of iNOS in lymphocytes is also enhanced by cytokines which induce H_2O_2 production, such as TNF-a and IL-1 [61]. Although nitric oxide can act as an antioxidant, the high production of nitric oxide induced by exposure to H_2O_2 could also enhance the reaction of this molecule with the superoxide anion, and then increase the levels of the highly toxic molecule peroxinitrite. The increase in nitrite levels however coexists with the activation of the superoxide detoxifying enzyme Mn-SOD, thus favoring the dismutation of superoxide to hydrogen peroxide and then avoiding peroxinitrite formation. Both iNOS and Mn-SOD genes participate of the same expression mechanism mediated by NF-kB [62].

All these antioxidant adaptations shown by the cells exposed to growing levels of H_2O_2 are however not enough to avoid the apparition of oxidative damage. Both proteins and DNA show clear evidences of oxidative damage in the cells treated with 1 and 10 nM H_2O_2/s. The comet assay is a sensitive method for detecting DNA damage at the level of individual cells, including detection of single and double strand breaks, incomplete excision repair sites and cross links. It has been proved to be a good marker of oxidative damage in the DNA of leukocytes [63-65]. Tail moment represents both the amount of DNA migrated into the tail and the distance migrated, and it has been reported as a valid marker of single-strand DNA breakage [66]. While the appearance of DNA oxidative damage appeared in the cells treated with 1 and 10 nM H_2O_2/s, the oxidation of proteins appeared even with the treatment with 0.1 nM H_2O_2/s, thus suggesting that proteins are more susceptible to oxidation than DNA in the presence of low levels of ROS. The DNA repairing enzymes could also be operating and could be sufficient to avoid the accumulation of excisions in DNA. Although the rapid activation of the antioxidant defenses (both at gene expression and activity levels) can not avoid the apparition of immediate oxidative damage, it could be of importance in avoiding pernicious effects on a long term basis in a preconditioning manner against subsequent ROS exposures, as it has been shown in other experimental designs which induce ROS production such as muscular contractions [67], hypoxia/reoxygenation [68] and hyperbaric oxygen treatments [44].

Taken together, these results show that when exposed to extracellular sustained physiological levels of H_2O_2, HL60 cells respond in a dose-response manner to the H_2O_2 concentration by activating the expression and activity of the antioxidant machinery, both cytoplasmic and

mitochondrial. However, growing levels of ROS also induce higher capacity to produce endogenous H_2O_2, and the activation of the antioxidant defenses is not enough to avoid the apparition of oxidative damage in proteins and DNA. For the experimental interest, of the two designs proposed the continuous exposure seems to be more appropriate to study the antioxidant response of HL60 cells to H_2O_2, because ROS are produced physiologically under relatively sustained mechanisms rather than in an isolated manner. Under an isolated bout of H_2O_2 the cell seems to activate the antioxidant mechanisms to rapidly reduce the increased levels of H_2O_2. To find an adequate acute dose of H_2O_2 to induce an adaptative response seems difficult because an initial dose as high as 100 μM has immediate toxic effects while low initial doses as 1 μM are rapidly consumed by the antioxidant defenses of the cells and no response is induced. However, the cell adapts to sustained H_2O_2 production by increasing the expression and activity of antioxidant enzymes, in a parallel manner to the apparition of oxidative damage. The experimental design using glucose/glucose oxidase to produce hydrogen peroxide in a sustained manner allows reproducing *in vitro* the effects of hormesis of the ROS.

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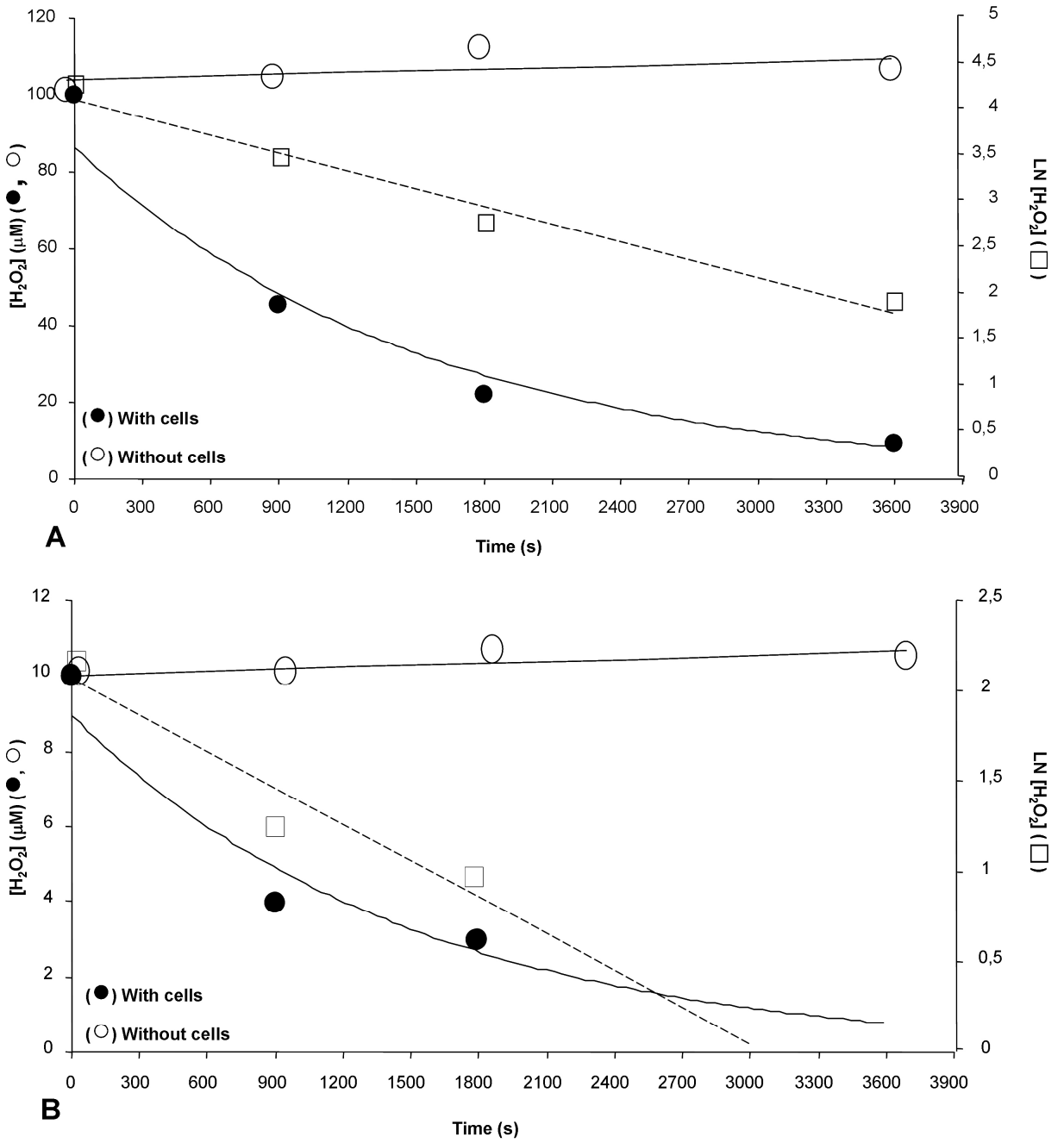
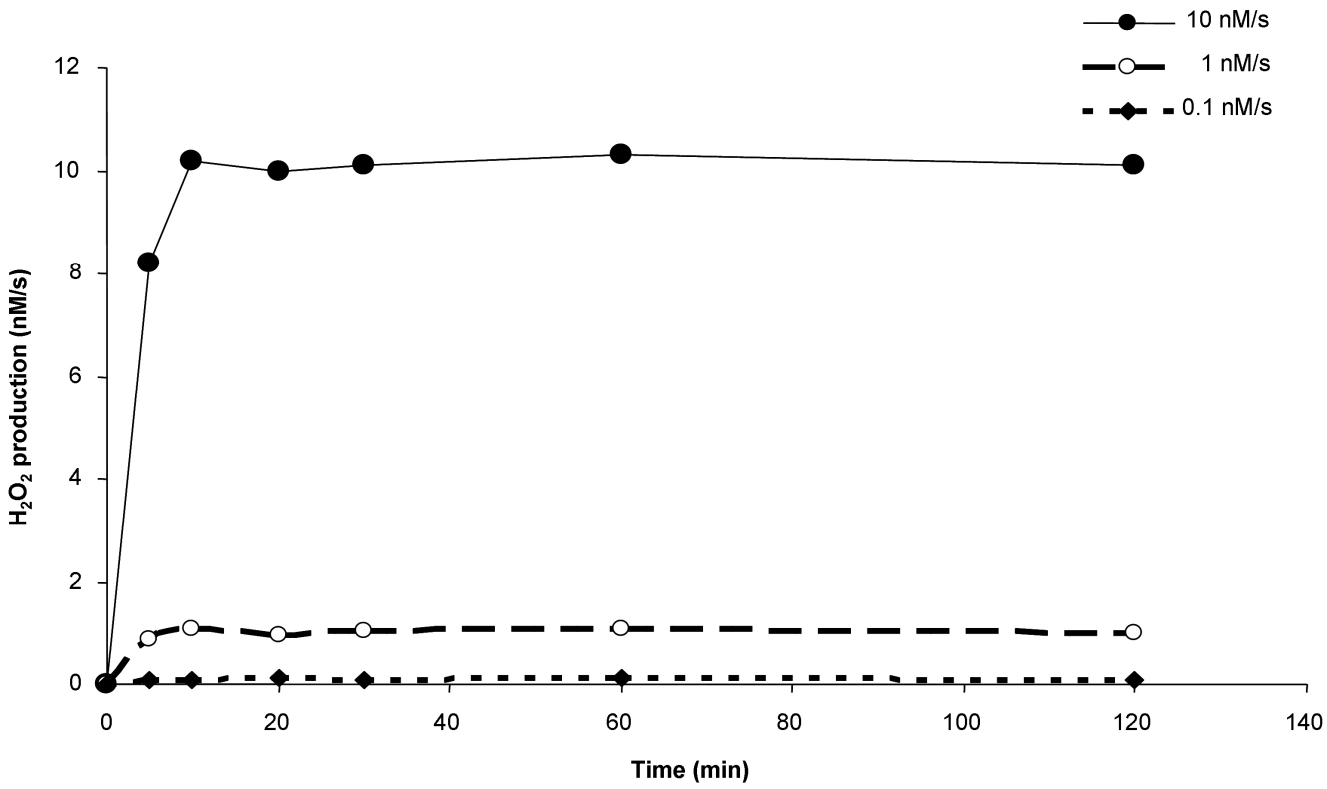
FIGURES

Figure 1. Changes in H_2O_2 concentrations with time.



The change in concentration of H_2O_2 in the culture medium both in the presence (●) and absence (○) of cells after the addition of (A) 100 μM and (B) 10 μM H_2O_2 was monitored colorimetrically using horseradish peroxidase and tetramethylbenzidine (TMB). The kinetic constants (k) for the disappearance of H_2O_2 were 15.8 and 12.2 k/10^9 cells after the addition of 10 and 100 μM H_2O_2, respectively.

Figure 2. H_2O_2 production with the glucose/glucose oxidase system



H_2O_2 concentration was measured in RPMI culture at time 0, 5, 10, 20, 30, 60 and 120 min after the addition of glucose oxidase and glucose. The rate of H_2O_2 production was maintained stable along time with the three different enzyme concentrations.

IV. RECAPITULACIÓN

1. Adaptaciones antioxidantes al ejercicio físico y a la hiperbaria

El desequilibrio entre la producción de especies reactivas de oxígeno y su eliminación induce estrés oxidativo. Tanto el ejercicio físico, en el cual las células incrementan el consumo de O_2 para hacer frente a la fuerte demanda energética, como la exposición a elevadas presiones de O_2 propias de la terapia hiperbárica o del buceo en botella a gran profundidad, son situaciones que afectan al equilibrio oxidativo. En estas situaciones se expone a la célula a niveles incrementados de O_2 y aumenta la producción de especies reactivas de oxígeno, llegando así a situaciones de desequilibrio y daño oxidativo. Hemos estudiado cómo estas situaciones de exposición aguda a incrementos en los niveles de O_2 podían afectar a la producción de especies reactivas de oxígeno, aparición de daño oxidativo y activación de las diferentes defensas antioxidantes en las diferentes compartimentos sanguíneos, tanto celulares como extracelulares. Así, hemos estudiado las adaptaciones de las células del sistema inmunitario (neutrófilos y linfocitos), de los eritrocitos y del plasma frente a incrementos agudos de la producción de especies reactivas de oxígeno.

La práctica de un ejercicio intenso o de larga duración provoca una respuesta inmunitaria similar a la respuesta de fase aguda, caracterizada por el incremento del número de neutrófilos circulantes y la activación de éstos [230, 231]. La neutrofilia se produce después de una sesión de natación, una etapa ciclista y un partido de entrenamiento de fútbol, así como después de una sesión de buceo con botella. Sin embargo, esta neutrofilia no se induce después de una sesión de terapia hiperbárica en reposo, lo que indica que la neutrofilia detectada tras la sesión de buceo es debida principalmente a la actividad física, o la combinación de actividad física e hiperbaria, más que a la hiperbaria por sí misma. La respuesta del neutrófilo al ejercicio no sólo se limita al incremento de su número en circulación, sino que a nivel intracelular se producen toda una serie de adaptaciones que parecen destinadas a la protección y reparación del tejido dañado. La actividad física produce en el neutrófilo incrementos en la actividad del enzima mieloperoxidasa (MPO), responsable de la producción de hipoclorito con función antimicrobiana, y en la producción de especies reactivas de oxígeno tras estimulación con el agente zymosan. La activación del neutrófilo conduce al ensamblaje de la NADPH oxidasa en las membranas peroxisomales y al inicio de la denominada explosión oxidativa, en la que se produce un incremento abrupto de la producción de especies reactivas de oxígeno [4]. Estas adaptaciones que producen incrementos de producción de ROS en el neutrófilo se han evidenciado después de una etapa ciclista en terreno llano de 240 minutos y después de un partido de fútbol y una sesión de natación de 60 minutos. Sin embargo, la intensidad del ejercicio

realizado influye en la respuesta de fase aguda del neutrófilo. El estudio de diferentes grupos experimentales que trabajan a diferente intensidad y tiempos similares durante un partido de fútbol nos ha permitido describir que esta respuesta de fase aguda del neutrófilo es altamente dependiente de la intensidad del ejercicio, ya que la magnitud del incremento en el recuento de neutrófilos depende de la intensidad del ejercicio realizado, a la vez que la activación de la producción de ROS sólo tiene lugar cuando el ejercicio realizado es de la suficiente intensidad. Todos estos cambios detectados en respuesta al ejercicio (neutrofilia, mayor actividad MPO y mayor producción de ROS) podrían desembocar en una situación de desequilibrio oxidativo, tanto en el propio neutrófilo como en los tejidos o medios circundantes. El incremento en la producción de ROS por el neutrófilo podría contrarrestarse con un incremento de las defensas antioxidantes endógenas y exógenas. Por este motivo, estudiamos las defensas antioxidantes y la aparición de daño oxidativo en el neutrófilo. Los neutrófilos se muestran bastante resistentes a la aparición de daño oxidativo tanto en lípidos como en proteínas [240, 312]. Únicamente una sesión de natación de una hora trabajando al 80% de la VO_2max es capaz de inducir un incremento en los niveles de malondialdehído (MDA) en neutrófilos, mientras que una etapa ciclista y un partido de fútbol no inducen la aparición de daño oxidativo. Sin embargo, estos resultados contrastan con el hecho de que, en los diferentes modelos estudiados, se aprecian descensos de las actividades catalasa, glutatión peroxidasa y superóxido dismutasa, mientras que simultáneamente en algunos de estos enzimas se detecta una activación de su expresión génica. Con el fin de profundizar en los mecanismos que podrían estar implicados en estos resultados aparentemente contradictorios de daño oxidativo, niveles de expresión y actividad enzimática nos centramos en el funcionamiento de la catalasa en el neutrófilo y sus adaptaciones frente al ejercicio intenso. La actividad catalasa disminuye en el neutrófilo después del ejercicio, mientras que sus niveles de expresión se incrementan. Cabe la posibilidad de que la catalasa sea secretada por el neutrófilo de forma similar a como se secreta la MPO como parte de la respuesta de fase aguda al ejercicio. La localización de la catalasa por inmunocitoquímica ha permitido esclarecer que antes del ejercicio la catalasa se encuentra localizada principalmente en forma soluble en el citoplasma, aunque también está presente en compartimentos vesiculares del neutrófilo. Tras la etapa ciclista detectamos un descenso en los niveles citoplasmáticos de catalasa y la ausencia de esta proteína en vesículas, por lo que se refuerza la idea de una cierta secreción de catalasa fuera del neutrófilo. Esta función ha sido respaldada mediante un estudio *in vitro* en el que la activación del neutrófilo con zymosan incrementa las actividades mieloperoxidasa y catalasa en el sobrenadante respecto a los valores previos a la activación. Estos

resultados apuntan a una posible secreción controlada de enzima catalasa del neutrófilo al medio extracelular durante la respuesta de fase aguda al ejercicio (de forma similar a la secreción de mieloperoxidasa), quizá con el fin de mantener controlados los niveles de especies reactivas en esta situación de producción incrementada de ROS. Este mecanismo parece ser operativo en todas las experiencias en las que se ha realizado una actividad física intensa, se ha inducido neutrofilia y un descenso de las actividad catalasa en el neutrófilo. Aparentemente, esta disminución de las actividades antioxidantes del neutrófilo podría incrementar la situación de estrés oxidativo e inducir daño oxidativo. Sin embargo, tan sólo en escasas circunstancias hemos apreciado daño oxidativo en neutrófilos. El neutrófilo puede disponer de otros enzimas antioxidantes que puedan protegerlo frente a la acción de las ROS, como la tioredoxina reductasa, o bien disponer de un mayor acervo de antioxidantes de bajo peso molecular como el ácido ascórbico o el glutatión. La respuesta inmunitaria del neutrófilo a la inmersión con botella a gran profundidad contrasta con la observada en los diferentes modelos de actividad física. Después de una sesión de buceo a 40 m de profundidad, la actividad MPO y la explosión oxidativa disminuyen en el neutrófilo. Parecer ser que la combinación de hiperoxia y actividad física, consecuencia de el buceo con botella, puede inhibir la maquinaria oxidativa del neutrófilo con tal de reducir la sensibilidad a estímulos inmunitarios, evitando de esta forma respuestas autoinmunes y facilitando la reparación muscular. Resultados similares se han obtenido en estudios previos en los que se evidencia que la práctica repetida de sesiones de buceo en apnea durante días consecutivos induce también una disminución en la explosión oxidativa del neutrófilo, lo que podría contribuir a la reparación muscular [376]. En resumen, la práctica de actividad física intensa induce en el neutrófilo una respuesta inmune prooxidativa caracterizada por la activación de la mieloperoxidasa y la explosión oxidativa. Esta respuesta oxidativa del neutrófilo se ve acompañada de una disminución de las defensas antioxidantes enzimáticas intracelulares, que parecen consecuencia, al menos en el caso de la catalasa, de una secreción controlada de enzima al plasma. El desequilibrio generado entre producción y eliminación de especies reactivas de oxígeno en el neutrófilo en respuesta al ejercicio físico no suele conducir, salvo excepciones, a la aparición de daño oxidativo.

Uno de los efectos que produce un ejercicio de alta intensidad y larga duración es una disminución del número de linfocitos circulantes tras un incremento inicial de los mismos durante el ejercicio [243]. Estos cambios en los linfocitos circulantes se relacionan con la aparición de un periodo con mayor susceptibilidad de padecer infecciones, principalmente de las vías respiratorias altas, denominándose a este fenómeno periodo de “ventana abierta” post-ejercicio [309, 377]. En los modelos de

ejercicio físico estudiados no hemos detectado cambios significativos en el número de linfocitos circulantes. No detectamos variación en el recuento linfocitario tras la sesión de buceo con botella, lo que indica que el ejercicio realizado durante la inmersión fue de una intensidad moderada, ni tras la exposición en reposo a oxígeno hiperbárico, indicando que la exposición a niveles incrementados de oxígeno no es suficiente para modificar el número de linfocitos circulantes. Un partido de entrenamiento de fútbol y una sesión de natación tampoco modifican significativamente el recuento de linfocitos, aunque en ambos casos se observa una tendencia a su disminución. Estos resultados, junto con los anteriormente descritos en neutrófilos, indican que la respuesta inmune al ejercicio se inicia con un incremento del número circulante de neutrófilos antes que con un descenso del número de linfocitos. Si la intensidad del ejercicio o su duración son las suficientes, la respuesta inmune continúa con la linfopenia, pero en los modelos de exposición aguda a ROS estudiados en esta tesis no la hemos detectado en ningún momento.

La producción de especies reactivas de oxígeno por parte del linfocito se ve claramente influenciada por el ejercicio físico y la exposición a niveles incrementados de oxígeno. Hemos detectado una mayor producción linfocitaria de ROS después de la sesión de buceo con botella, después de la sesión de natación y después del partido de fútbol de entrenamiento. A diferencia de los neutrófilos, que necesitan activarse con zymosan para producir especies reactivas, los linfocitos producen ROS sin necesidad de activación inmunológica previa, mientras. Al igual que en el neutrófilo, el incremento en la producción de ROS en el linfocito depende de la intensidad del ejercicio realizado, ya que en el partido de fútbol sólo detectamos este incremento en el grupo que trabajó a mayor intensidad. La estimulación con PMA induce un estado de activación del linfocito en el que parecen estar implicadas las rutas de señalización de la proteína quinasa C (PKC), que conducen finalmente a un incremento en la producción de especies reactivas. Por tanto, lo que se determina tras la activación con PMA no es tanto la producción basal de ROS como la susceptibilidad del linfocito a producir ROS en condiciones de activación. En los tres modelos de ejercicio utilizados, así como tras la exposición en reposo a oxígeno hiperbárico, detectamos un incremento en la producción de ROS tanto en los linfocitos no activados como en los activados con PMA, indicando que no sólo aumenta su capacidad de producción de ROS sino también la producción basal. Es importante reseñar que a diferencia de lo observado en el neutrófilo, la exposición a oxígeno hiperbárico es por sí sola suficiente para incrementar la producción linfocitaria de ROS, manifestando así la importancia de la tensión de oxígeno en la producción celular de especies reactivas.

La mayor producción de especies reactivas por parte del linfocito coexiste con la activación de las defensas antioxidantes enzimáticas. En vista de los resultados obtenidos con los diferentes modelos utilizados, la glutatión peroxidasa parece ser uno de los enzimas activados con mayor presteza en el linfocito en respuesta al estrés oxidativo, si bien la catalasa y, principalmente, la superóxido dismutasa también se ven activadas en respuesta al ejercicio. Sin embargo, los mecanismos de regulación de estas defensas antioxidantes enzimáticas difieren entre ellos. En ninguno de los casos en que hemos detectado un incremento en la actividad glutatión peroxidasa hemos detectado variaciones en los niveles de expresión del gen, lo que indica que la respuesta de este enzima se debe a mecanismos post-transcripcionales. En el caso de la SOD, sin embargo, sí que hemos detectado un incremento en los niveles de expresión del gen Mn-SOD paralelo al incremento en su actividad enzimática tras la sesión de natación. Aunque ni la actividad ni la expresión de la catalasa llegan a variar significativamente después de una sesión de natación, detectamos una fuerte correlación positiva entre los niveles de expresión del PGC-1α, la sirtuina 3 (SIRT3) y la catalasa. El PGC-1α es un coactivador transcripcional que potencia la expresión de genes involucrados en la biogénesis mitocondrial y el desacoplamiento de la cadena respiratoria, pero también de genes involucrados en la respuesta antioxidante como la Mn-SOD y la catalasa. Aunque los mecanismos por los que se produce la activación del PGC-1α en respuesta al ejercicio no están del todo definidos, en vista de las correlaciones obtenidas y resultados previos de otros investigadores, la SIRT3 podría estar implicada en la desacetilación y activación del PGC-1α en linfocitos. La hemooxigenasa 1 (HO-1) es otra de las defensas antioxidantes que se ha visto que responde habitualmente a diversas situaciones de estrés oxidativo aumentando su expresión. La importancia de la HO-1 como defensa antioxidante es atribuida a la producción de bilirrubina (producto de la degradación del grupo hemo), un reconocido antioxidante celular, y al incremento en el secuestro de hierro debido a los niveles aumentados de ferritina. Estudios previos pusieron de manifiesto que la expresión de este gen se ve inducida tras una exposición hiperbárica [149, 153]. En nuestro estudio observamos esta misma inducción tras la terapia hiperbárica pero también después de la sesión de buceo con botella. En cuanto al ejercicio físico, detectamos la activación del gen de la HO-1 tras el partido de fútbol en los grupos de intensidad media y alta pero no en el grupo de baja intensidad, evidenciando así la importancia de la HO-1 como defensa antioxidante activada en respuesta al estrés oxidativo.

A pesar de que en ninguno de los modelos estudiados detectamos una reducción significativa del número de linfocitos circulantes, tras la sesión de natación

se produce un descenso en la expresión de la proteína Bcl-2 de alrededor del 50%. La Bcl-2 es una proteína antiapoptótica que participa en la regulación de procesos apoptóticos. Debido a su estructura y localización en la membrana mitocondrial, junto con su función antiapoptótica podría ser importante a la hora de regular el flujo protónico y el potencial de membrana en la mitocondria, actuando así sobre la producción de especies reactivas de oxígeno. Por ello la Bcl-2 está también considerada como una proteína antioxidante. Junto con el descenso en la expresión de la Bcl-2 se produce también un descenso en la expresión de la proteína UCP-3 tras una sesión natación, y se observa una correlación entre la expresión de ambos genes después del ejercicio. Esta regulación negativa de la UCP-3 en respuesta al ejercicio agudo es especialmente llamativa teniendo en cuenta los resultados que se han obtenido hasta el momento en músculo esquelético, donde la expresión de la UCP-3 se ve activada tras realizar un ejercicio puntual. Nuestros resultados apuntan a una regulación diferencial de la UCP-3 en el músculo esquelético y el linfocito. En este último tipo celular, el incremento de ROS asociado al ejercicio podría disminuir la expresión de los genes UCP-3 y Bcl-2, comprometiendo de esta forma una de las vías de defensa antioxidante y potenciando la situación de estrés oxidativo. La existencia de una correlación negativa entre las expresiones de UCP-3 y Bcl-2 y los niveles de MDA apunta a esta desactivación de estas defensas antioxidantes paralela la aparición de daño oxidativo. La inactivación de la Bcl-2 podría estar involucrada también en la entrada en un proceso apoptótico, aunque los datos obtenidos son insuficientes como para aseverarlo.

Los niveles de nitrito como marcador del óxido nítrico (NO) y de la proteína responsable de su síntesis en el linfocito, la iNOS, responden de forma similar en los diferentes modelos de actividad física estudiados. Tanto los niveles de nitrito como los de iNOS aumentan durante el período de recuperación tras una inmersión con botella y tras una sesión de natación. Aunque una exposición hiperbárica en reposo no induce un incremento significativo de los niveles de iNOS, la tendencia a aumentar es bastante evidente y la diferencia puede ser debida a que el aumento significativo tras el buceo se evidencia 3 horas después de haber finalizado la inmersión, mientras que las muestras de la cámara hiperbárica se obtuvieron inmediatamente después de la exposición. Se sabe que el óxido nítrico puede producir tanto efectos beneficiosos como perjudiciales dependiendo de su concentración [378]. Si su concentración no supera un determinado valor umbral, el NO juega un papel vital en la transducción de señales y contribuye al proceso de relajación muscular endotelial. Sin embargo, si su concentración excede el umbral, su contribución cambia y facilita el daño celular a través de la formación de compuestos como el peroxinitrito, que es altamente tóxico y

produce muerte celular por nitración e inactivación de moléculas esenciales (proteínas, DNA, etc.) [379]. Algunos estudios han evidenciado la existencia de estrés oxidativo y niveles aumentados de NO en neutrófilos circulantes en enfermedades neurodegenerativas como el Alzeheimer y el Parkinson, relacionando este hecho con el desarrollo de la enfermedad [380, 381]. Nosotros hemos encontrado asociaciones directas entre las expresiones linfocitarias de iNOS y Mn-SOD, indicando que estos genes responden a los mismos estímulos. A la vez, se pone de manifiesto la necesidad de controlar la producción de peroxinitrito, producto de la reacción entre el óxido nítrico y el anión superóxido, ya que paralelamente al incremento de la capacidad de producción de NO se produce un incremento de la capacidad de eliminación del anión superóxido. Tanto la actividad física intensa como la exposición a oxígeno en condiciones de hiperbaria incrementan la expresión linfocitaria de iNOS, el cual a su vez incrementa la producción de NO. A la vez, se produce un incremento en la actividad o expresión de Mn-SOD con objeto de evitar los posibles efectos perjudiciales asociados al incremento de la producción de NO. Entre los efectos que tiene el NO sobre el linfocito se encuentran la estimulación de la biogénesis mitocondrial a través de la activación del PGC-1α, estimulación o inhibición de la apoptosis y regulación de las vías de transducción de señal [382].

Evidenciada la mayor producción linfocitaria de especies reactivas de oxígeno asociada al ejercicio y la hiperbaria y la activación de las defensas antioxidantes en el linfocito, la última fase de este estudió consistió en determinar si se producía un desequilibrio oxidativo capaz de conducir a la aparición de daño oxidativo en las biomoléculas celulares. Los niveles de MDA no se ven modificados como consecuencia de la exposición a hiperbaria ni tras la práctica de natación. Un partido de fútbol tampoco induce incrementos en los niveles linfocitarios de MDA en los individuos que participan a una intensidad baja y media. Sin embargo, en los individuos que trabajan a alta intensidad durante el partido, se induce incremento de la peroxidación lipídica. Por tanto, podemos decir que la respuesta antioxidante del linfocito al ejercicio físico se muestra en general efectiva a la hora de prevenir la aparición de daño oxidativo, aunque si el ejercicio es de la suficiente intensidad la producción de ROS sobrepasa las defensas antioxidantes y conlleva la aparición de daño oxidativo. Al realizar un estudio de correlaciones observamos que los niveles de MDA después del ejercicio están positivamente correlacionados con la actividad de los enzimas catalasa y glutatión peroxidasa, evidenciando así la estrecha relación que se establece entre la activación de las defensas antioxidantes y la aparición de daño oxidativo.

La situación oxidativa del plasma es considerada como un buen marcador del balance oxidativo global del organismo, por lo que también estudiamos el estado de las defensas antioxidantes y los marcadores de daño oxidativo en este compartimiento extracelular. Los niveles plasmáticos de LDH y CK se utilizan como marcadores de daño y lisis celular, especialmente muscular y eritrocitaria. Tanto el buceo como la etapa ciclista inducen incrementos en los niveles séricos de ambos marcadores de lisis, y la misma tendencia se observa tras la práctica de la natación. Cabe señalar que no se observan cambios en los niveles de LDH y CK tras la exposición en la cámara hiperbárica, lo cual descarta la elevada tensión de oxígeno como responsable de este daño celular y apunta más al desgaste provocado por la actividad física. En cuanto a las defensas antioxidantes enzimáticas observamos un incremento de actividad de la catalasa y la superóxido dismutasa en plasma tras la inmersión con botella. Los enzimas en plasma pueden provenir de diferentes fuentes celulares, pero estos resultados se muestran en consonancia con los mecanismos expuestos anteriormente según los cuales el neutrófilo secretaría enzimas antioxidantes al medio extracelular durante su proceso de activación en respuesta al ejercicio físico. Entre los antioxidantes nutricionales, observamos que los niveles de ascorbato aumentan tras la natación y se aprecia una tendencia similar aunque no significativa tras el partido de fútbol. El análisis de la respuesta plasmática al fútbol se realizó en un partido diferente al expuesto anteriormente para los linfocitos, y en este partido todos los jugadores trabajaron más del 50% de la duración del partido a un nivel superior al 80% de su capacidad máxima. La presencia en este caso de una tendencia no significativa podría ser el resultado de una respuesta diferencial en función del esfuerzo individual realizado por cada jugador. En conjunto, estos resultados muestran que las defensas antioxidantes del plasma se potencian en respuesta al ejercicio físico y la hiperbaria. Al igual que lo descrito para los linfocitos, esta activación de las defensas no es suficiente para evitar la aparición de daño oxidativo, ya que se inducen incrementos en los niveles de MDA después de la sesión de natación y el partido de fútbol y de los grupos carbonilo después de la etapa ciclista.

2. Adaptaciones diferenciales al estrés oxidativo en función del género

En los últimos años se ha puesto de manifiesto que la capacidad antioxidante es dependiente del género. Estudios realizados en animales evidencian que las hembras presentan una capacidad antioxidante mayor que los machos, relacionándose estas diferencias con una mayor longevidad femenina [356]. Las hormonas sexuales femeninas parecen tener un papel clave en el control de la

capacidad antioxidante celular [353]. La respuesta antioxidante endógena al ejercicio intenso puede verse de esta forma afectada por el género del deportista, repercutiendo de forma diferente en hombres y mujeres en cuanto a la aparición de daño oxidativo.

Los linfocitos de nadadoras presentan una mayor actividad basal del enzima Mn-SOD que los linfocitos de los nadadores, por lo que la capacidad basal de eliminación del anión superóxido producido a nivel mitocondrial es mayor en los linfocitos de las chicas que de los chicos. Sin embargo, en respuesta a una sesión de natación, la actividad Mn-SOD linfocitaria aumenta en los chicos pero se mantiene a nivel basal en las chicas, de forma que tras el ejercicio esta actividad se iguala entre ambos géneros. Así, los linfocitos de las nadadoras presentan de entrada una mayor protección antioxidante, que resulta similar a la que adquieren los linfocitos de nadadores después de un ejercicio intenso. Este incremento de actividad en los chicos en respuesta al ejercicio parece debido a un incremento en la expresión génica del enzima, incremento que no se detecta en las chicas. De forma paralela, la sesión de natación también incrementa la expresión del gen de la iNOS, que a su vez se ve reflejado en un incremento en los niveles linfocitarios de nitrito. Aunque no se detecta un efecto significativo del género en estos parámetros, cabe señalar que dicho incremento en la expresión de la iNOS sólo es significativo en el grupo de los chicos, sugiriendo así una mayor activación génica en chicos que en chicas. Las especies reactivas han sido postuladas como reguladoras de la expresión de determinados genes, entre ellos la Mn-SOD y la iNOS [360]. En este sentido, el género ejerce un efecto sobre la producción de especies reactivas de oxígeno y nitrógeno en el linfocito en respuesta al ejercicio. Así, si bien en condiciones basales la producción linfocitaria de ROS no difiere entre ambos géneros, la sesión de natación induce un incremento en la producción de ROS y RNS sólo en los chicos, que finalmente alcanzan mayores niveles de producción que las chicas. Experimentos con cultivos celulares han evidenciado que la activación de las defensas antioxidantes tales como la Mn-SOD o la catalasa en respuesta al tratamiento con H_2O_2 depende, al menos en parte, de la activación del cofactor transcripcional PGC-1α [383]. A su vez, el óxido nítrico también actúa como un inductor del PGC-1α [384]. En nuestro estudio observamos una interacción entre los factores ejercicio y género en la expresión del PGC-1α, de forma que tras el ejercicio los niveles de mRNA de PGC-1α tienden a subir en chicos, mientras que la tendencia en chicas es a disminuir. Estos resultados sugieren que los linfocitos de los chicos producen mayores niveles de especies reactivas de oxígeno y nitrógeno en respuesta al ejercicio, y estos niveles incrementados podrían activar las defensas antioxidantes vía activación del PGC-1α. Las chicas, por el contrario se

mostrarían más resistentes a la producción de especies reactivas tras el ejercicio y, por tanto, a la activación de las defensas antioxidantes. Sin embargo, las correlaciones entre la expresión de los diferentes genes coinciden en ambos géneros, por lo que los mecanismos que operan en la activación de las defensas antioxidantes parecen ser los mismos en ambos géneros.

La mayor protección antioxidante que parecen poseer, por lo general, las hembras respecto de los machos, ha sido parcialmente atribuida a las hormonas estrogénicas [353]. Por ello, determinamos los niveles plasmáticos de 17-β-estradiol y detectamos niveles mayores de la hormona en las chicas respecto de los chicos, como es de esperar en una población en edad fértil. Los chicos muestran una mayor respuesta inflamatoria y una situación de estrés oxidativo plasmático más marcada que las chicas después de realizar la actividad física, ya que tras el ejercicio tanto la actividad creatina quinasa (CK) y el recuento de neutrófilos (como marcadores de inflamación) como la actividad gamma-glutamil-transpeptidasa (GGT) y los niveles plasmáticos de MDA (como marcadores de estrés oxidativo) son mayores en chicos que en chicas. El 17-β-estradiol podría estar actuando de forma directa como un antioxidante, pero también al actuar como un antiinflamatorio prevendría la mayor producción de ROS asociada a los procesos inflamatorios.

En resumen, una sesión de natación de una hora al 80% de la capacidad máxima induce una mayor activación de las defensas antioxidantes linfocitarias en los chicos respecto de las chicas, pero también es mayor en los chicos la respuesta inflamatoria al ejercicio y el daño muscular y plasmático. La mayor protección de las chicas a la aparición de daño muscular y oxidativo parece estar relacionada con las propiedades antioxidantes y antiinflamatorias del 17-β-estradiol.

3. Estrés oxidativo asociado a la enfermedad de la porfiria variegata

La actividad física genera situaciones agudas de estrés oxidativo que, a base de su repetición durante los entrenamientos, pueden inducir adaptaciones duraderas en las defensas antioxidantes y frente al estrés oxidativo. La porfiria variegata, causada por deficiencias en la síntesis de porfirinas, puede considerarse como una situación de desequilibrio oxidativo crónico, con fases agudas de incrementos en la producción de radicales libres asociados a las crisis porfíricas. La porfiria variegata es una enfermedad resultado del deficiente funcionamiento del penúltimo enzima de la ruta de síntesis del grupo hemo, la protoporfirinógeno oxidasa (PPOX). La acumulación de porfirinas y sus precursores, especialmente el ácido δ-aminolevulínico

(δ-ALA), puede provocar daño oxidativo en diferentes biomoléculas a través de una acción directa o por la producción de especies reactivas de oxígeno, llegando a inducir una situación de estrés oxidativo. Pero junto con los efectos directos de estos compuestos intermediarios sobre la producción de ROS o daño oxidativo, la condición porfírica puede generar estrés oxidativo por otras vías. Debido a que el hemo es un constituyente esencial de un importante número de proteínas, la dificultad a la hora de sintetizar este grupo prostético podría estar relacionada con un funcionamiento limitado de estas proteínas. Así, las limitaciones en la síntesis de grupo hemo podrían afectar a la funcionalidad de componentes de la cadena respiratoria mitocondrial como son los citocromos, induciendo una mayor producción de ROS, o a la funcionalidad de enzimas antioxidantes como la catalasa, mermando la capacidad detoxificadora de éstos. Hemos determinado los efectos de la porfiria variegata sobre la producción endógena de especies reactivas de oxígeno, sobre los niveles de marcadores de daño oxidativo y sobre diferentes defensas antioxidantes, tanto enzimáticas como no enzimáticas, en los diferentes compartimentos sanguíneos, así como sobre diferentes marcadores de inflamación.

La deficiencia de PPOX en mujeres afectadas de porfiria variegata queda patente en eritrocitos y linfocitos. Los niveles de proteína PPOX en eritrocitos de mujeres con porfiria variegata son de aproximadamente el 50% de los niveles detectados en mujeres control. Paralelamente, en el linfocito encontramos una expresión génica de este enzima también reducida en las mujeres afectadas de porfiria variegata. A pesar de que los niveles eritrocitarios de PPOX están reducidos en mujeres porfíricas, los niveles de hemoglobina son mayores en estas mujeres, de forma que la síntesis de la hemoglobina no se ve perjudicada por el déficit de PPOx, al menos en condiciones basales. Sin embargo, hemos detectado la presencia de estrés oxidativo en los eritrocitos de mujeres afectadas de porfiria variegata, quienes presentan mayores niveles de MDA que las mujeres control. Así mismo, la disminuida actividad sanguínea δ-aminolevulínico dehidratasa (δ-ALAD) presente en mujeres porfíricas es una evidencia adicional de daño oxidativo asociado a la porfiria variegata, y se muestra de acuerdo con resultados previos según los cuales la acumulación de precursores del grupo hemo puede oxidar este enzima, afectando así a su actividad [281]. Esta situación de mayor producción de especies reactivas de oxígeno asociado a la porfiria variegata puede ser compensada mediante unas mayores actividades de los enzimas antioxidantes en eritrocitos. Las actividades de los enzimas antioxidantes glutatión reductasa y superóxido dismutasa eritrocitarias son mayores en mujeres porfíricas. Esta mayor actividad antioxidante endógena podría estar relacionada con una mayor síntesis de estos enzimas en los precursores celulares del eritrocito, si bien

en trabajos previos hemos puesto de manifiesto que para una cantidad concreta de enzima en el eritrocito, la presencia de mayores niveles de ROS puede potenciar directamente la actividad enzimática de la glutatión reductasa y la superóxido dismutasa [239, 385]. Así, las mayores actividades eritrocitarias que presentan estos enzimas asociados a la porfiria variegata podrían estar relacionadas con la exposición a niveles mayores de ROS en comparación con las mujeres control. Sin embargo, la actividad catalasa no sigue este patrón, ya que no se ve afectada por la enfermedad. Al analizar los niveles de proteína catalasa se pone de manifiesto que, a pesar de la actividad similar, los niveles de proteína son superiores en las mujeres afectadas de porfiria. Estos resultados sugieren que los eritrocitos de las mujeres con porfiria variegata tienen alterada la actividad catalasa como consecuencia de la síntesis limitada del grupo hemo. El balance entre una mayor activación de las defensas antioxidantes del eritrocito y la presumible mayor producción de ROS no parece suficiente como para contrarrestar la situación de estrés oxidativo, ya que se observan marcadores de daño oxidativo superiores en las mujeres afectadas por la enfermedad. Así, tanto los niveles eritrocitarios de MDA como el volumen corpuscular medio, ambos marcadores de estrés oxidativo, son mayores en mujeres porfíricas.

Los efectos de la porfiria variegata sobre el linfocito son diferentes a los observados en el eritrocito. En el linfocito la porfiria provoca una disminución de la actividad de los enzimas catalasa y glutatión peroxidasa. La expresión génica de la catalasa no se ve afectada por la enfermedad, lo que apunta a mecanismos de regulación post-transcripcionales. Al igual que lo observado en el eritrocito, la limitación en la síntesis del grupo hemo podría influir en la disponibilidad de catalasa enzimáticamente activa. En la glutatión peroxidasa, por el contrario, sí que detectamos también una disminución en la expresión génica del enzima en las mujeres afectadas de porfiria. Otras proteínas cuyas funciones han sido también descritas como antioxidantes, tales como la UCP-3, Bcl-2 y SIRT3, presentan expresiones reducidas en mujeres porfíricas en comparación con el grupo de mujeres control. De esta forma, resulta evidente que los linfocitos de mujeres afectadas de porfiria variegata presentan una alteración de las diferentes defensas antioxidantes. Esta menor actividad o expresión de las defensas antioxidantes no resulta, sin embargo, en una mayor situación de estrés oxidativo a nivel basal, en base a los niveles mantenidos de MDA e índice de carbonilos.

La producción de especies reactivas de oxígeno tampoco difiere en condiciones basales entre mujeres afectadas de porfiria variegata y mujeres sanas. Sin embargo, tras la estimulación con PMA, los linfocitos de mujeres porfíricas producen mayores cantidades de H_2O_2 que los linfocitos de mujeres control. El

tratamiento *in vitro* de los linfocitos con inhibidores específicos de diferentes fuentes productoras de ROS apunta a la cadena respiratoria mitocondrial, y más concretamente al complejo III, como la posible fuente de esta mayor producción de ROS en mujeres porfíricas. En base a estos resultados, estudiamos el daño por roturas en el DNA en condiciones basales y tras el tratamiento *in vitro* de los linfocitos con H_2O_2. De acuerdo con los resultados obtenidos de MDA e índice de carbonilos, el nivel de daño en el DNA es equivalente en mujeres control y porfíricas en condiciones basales. Sin embargo, tras el tratamiento con H_2O_2 los linfocitos obtenidos de mujeres porfíricas presentan mayores niveles de rotura en el DNA. Cogidos en conjunto, todos estos resultados evidencian que los linfocitos de mujeres afectadas de porfiria variegata presentan una disminución de ciertas defensas antioxidantes como la catalasa, glutatión peroxidasa, UCP-3, Bcl-2 y SIRT3, pero esta menor capacidad antioxidante no resulta en una situación de estrés oxidativo linfocitario en condiciones basales, a diferencia de lo observado en el eritrocito. Sin embargo, los linfocitos de mujeres porfíricas son más susceptibles a producir especies reactivas de oxígeno y sufrir daño oxidativo cuando están sometidos a situaciones de estrés tales como la activación inmunitaria o el tratamiento con H_2O_2, respectivamente.

Determinadas enfermedades en las que se ven implicados de forma directa o indirecta niveles incrementados de especies reactivas de oxígeno han sido a menudo relacionadas con procesos proinflamatorios. Las mujeres afectadas de porfiria variegata presentan un estado de leve inflamación crónica cuando las comparamos con mujeres control de la misma edad. La presencia de un mayor número de neutrófilos y monocitos circulantes, así como de niveles plasmáticos incrementados de proteína C reactiva, pone de manifiesto esta situación de inflamación padecida por estas mujeres. El número incrementado de neutrófilos circulantes en mujeres porfíricas va acompañado de un mayor estado de activación de estos neutrófilos, evidenciado por la mayor capacidad de producción de ROS de éstos en respuesta a su estimulación inmunitaria. Esta mayor susceptibilidad del neutrófilo a la producción de ROS en mujeres porfíricas coexiste con una disminución de las defensas antioxidantes enzimáticas, concretamente la catalasa y la glutatión reductasa, lo que repercute en unos mayores niveles de oxidación proteica. Así pues, la condición porfírica afecta de forma similar a linfocitos y neutrófilos, induciendo en ellos una cierta inactivación de las defensas antioxidantes enzimáticas. Si bien en el linfocito esta deficiencia no conlleva la aparición de daño oxidativo en condiciones basales, en el neutrófilo sí que aparece daño oxidativo en condiciones basales.

4. Efectos potenciadores de nutrientes antioxidantes sobre las defensas antioxidantes endógenas y la aparición de daño oxidativo

Queda patente con los resultados expuestos hasta el momento que determinadas situaciones que suponen una exposición a altos niveles de especies reactivas de oxígeno o de alta producción de éstas pueden llevar a la instauración de una situación de estrés oxidativo, lo que puede repercutir de forma global en el estado de salud de las personas que lo padecen. Durante muchos años se llevan estudiando diferentes intervenciones dietéticas con nutrientes antioxidantes con el fin de tratar de establecer una terapia nutricional que reduzca el estrés oxidativo a través de la potenciación de las defensas antioxidantes. La suplementación con altas dosis de vitaminas y otros nutrientes antioxidantes, a pesar de mostrarse efectiva a la hora de incrementar los niveles tisulares de estos nutrientes y de otorgar protección antioxidante, también puede interferir en la respuesta endógena celular al estrés oxidativo, ya que estos antioxidantes actuarían impidiendo que las especies reactivas de oxígeno o determinados productos de oxidación actuaran como los señalizadores celulares de activación de las defensas endógenas. Es por tanto evidente la necesidad de encontrar los nutrientes y sus dosis adecuados para optimizar el estatus antioxidante de la célula, protegiéndola frente a la aparición de daño y estrés oxidativo, pero evitando a la vez la interferencia en la respuesta adaptativa celular.

Estudiamos tres tipos de suplementaciones con antioxidantes diferentes, con la presencia común de las vitaminas C y E en todos ellos, aunque a dosis diferentes y en combinación con otros nutrientes. En uno de los estudios, utilizamos un suplemento que contenía únicamente vitaminas C y E, en unas dosis de 2,5 y 5 veces las CDR para estas vitaminas, respectivamente. En otro estudio utilizamos este suplemento, pero adicionalmente enriquecido con un extracto de fitoestrógenos, que incrementa aún más los niveles de antioxidantes ingeridos. Finalmente, en otro estudio utilizamos un cocktail multivitamínico y mineral, en el que las vitaminas C y E se suministraron en dosis del 100% de las CDR, y adicionalmente enriquecido con coenzima Q. Por tanto, las dosis utilizadas de vitaminas antioxidantes no fueron excesivamente elevadas, con el fin de intentar que no interfirieran en la respuesta endógena de las células. Los modelos utilizados para generar estrés oxidativo o una respuesta antioxidante que utilizamos reflejan situaciones de estrés oxidativo agudo (fútbol y natación) o crónico (porfiria variegata). Los diferentes suplementos utilizados se muestran ciertamente efectivos a la hora de incrementar la protección de los individuos suplementados frente al estrés oxidativo, ya sea a través de la activación de las defensas antioxidantes enzimáticas o a través de la disminución de los niveles de marcadores de daño

oxidativo, y en ningún caso modifican la respuesta antioxidante endógena de las células al estrés oxidativo.

En el primer estudio de suplementación analizamos los efectos de la ingesta durante 3 meses de un cocktail multivitamínico y mineral (composición en la Tabla 3) sobre las defensas antioxidantes y la aparición de daño oxidativo en futbolistas pre-profesionales, en condiciones basales y tras la práctica de un partido de fútbol. Entre otros compuestos, este suplemento contenía 60 mg de vitamina C (100% de la CDR), 10 mg de vitamina E (100% de la CDR) y 100 mg de coenzima Q. Estos 3 meses de suplementación coincidieron con un período de entrenamiento de los deportistas, por lo que a la vez estudiamos los efectos del entrenamiento del fútbol sobre las defensas antioxidantes y el daño oxidativo, así como los efectos del suplemento sobre la respuesta endógena al entrenamiento. La suplementación durante 3 meses con este cocktail es suficiente como para incrementar los niveles basales plasmáticos de coenzima Q. Los niveles de ascorbato y α-tocoferol, sin embargo, no aumentan en plasma, linfocitos ni neutrófilos como consecuencia de dicha suplementación. En el linfocito observamos una adaptación de los sistemas antioxidantes durante el período de entrenamiento, con un descenso de la actividad catalasa y un incremento de la actividad glutatión reductasa, así como con un descenso en los niveles linfocitarios de α-tocoferol. Estos cambios en las defensas antioxidantes se ven acompañados de un descenso en los niveles de MDA, indicando que las adaptaciones ocurridas durante el entrenamiento conducen a una mayor protección del linfocito frente al estrés oxidativo. En el neutrófilo observamos un descenso en los niveles intracelulares de ascorbato al final del período de entrenamiento, y una disminución de la actividad glutatión reductasa, mientras que los niveles de MDA se mantienen a nivel basal. En el plasma, a diferencia de lo observado en las células, se genera un aumento en los niveles de MDA al final del período de entrenamiento, indicando la aparición de daño oxidativo plasmático como consecuencia del ejercicio crónico. La suplementación con el cocktail multivitamínico y coenzima Q no modifica las respuestas endógenas del linfocito ni del neutrófilo al entrenamiento, pero tampoco consigue prevenir el incremento del daño oxidativo en plasma durante este período.

Tras los 3 meses de suplementación, los futbolistas jugaron un partido de fútbol de entrenamiento de 60 minutos, similar al que se jugó antes del período de entrenamiento y suplementación. El ejercicio realizado durante un partido de fútbol tras un período de entrenamiento de 3 meses no es suficiente como para activar los enzimas antioxidantes en linfocitos, pero sí para incrementar la capacidad linfocitaria de producción de ROS y los niveles de peroxidación lipídica. La suplementación con vitaminas y coenzima Q no es capaz de evitar la aparición de daño oxidativo en

linfocitos como consecuencia del partido. Por el contrario, en el neutrófilo sí que se produce una respuesta antioxidante a un partido de fútbol, reflejada en una disminución en la actividad de los enzimas catalasa y glutatión peroxidasa tras el ejercicio, como se ha descrito en anteriores ocasiones tras realizar diferentes ejercicios. Los niveles intracelulares de α-tocoferol en el neutrófilo también disminuyen tras el partido de fútbol, mientras que los niveles de MDA permanecen invariables. La suplementación con dosis bajas de vitaminas antioxidantes y coenzima Q no interfiere en esta respuesta antioxidante del neutrófilo a la actividad física. En el plasma, por el contrario, la suplementación con vitaminas y coenzima Q ejerce un efecto sobre los marcadores de daño oxidativo, de forma que después de jugar el partido los individuos suplementados presentan menores niveles de MDA y grupos carbonilo que los sujetos placebo. Por tanto, la suplementación de la dieta con vitaminas antioxidantes en concentraciones de aproximadamente el 100% de las CDR y con coenzima Q no modifica la respuesta endógena al entrenamiento de fútbol y a la práctica de un partido de fútbol, pero sí otorga una mayor protección frente a la aparición de daño oxidativo en lípidos y proteínas en plasma tras sesiones agudas de ejercicio físico como pueda ser un partido de fútbol.

En el segundo estudio de suplementación utilizamos la condición de porfiria como modelo de estrés oxidativo para estudiar los efectos de una bebida de almendra enriquecida con vitaminas E y C, de forma que los individuos suplementados ingirieron 50 mg de vitamina E (500% de la CDR) y 150 mg de vitamina C (250% de la CDR) al día durante 6 meses. Este estudio de suplementación no lo llevamos a cabo únicamente con pacientes de porfiria, sino también con las mujeres control sanas que utilizamos en el estudio previo para caracterizar la porfiria variegata como una enfermedad prooxidante. De esta forma analizamos no sólo si la bebida enriquecida con vitaminas se mostraba efectiva a la hora de mejorar una situación ya de por sí prooxidativa sino también si mejoraba las defensas antioxidantes de una población control no sometida a una situación de estrés oxidativo. El consumo de la bebida suplementada induce un incremento en los niveles de α-tocoferol en linfocitos y neutrófilos, tanto en mujeres porfíricas como en mujeres control. Sin embargo, los niveles de ascorbato en el linfocito y el neutrófilo no se ven significativamente afectados por la suplementación. A su vez, el consumo incrementado de vitaminas C y E induce un descenso en los niveles plasmáticos de productos de peroxidación lipídica, tanto en mujeres porfíricas como en sus controles. En linfocitos, la suplementación con antioxidantes induce una activación post-transcripcional de la glutatión reductasa y la superóxido dismutasa, tanto en mujeres porfíricas como controles, pero sin afectar a los niveles de marcadores de oxidación molecular como

los carbonilos y el MDA. La mayor producción de especies reactivas de oxígeno por parte de los linfocitos de mujeres afectadas de porfiria tras su activación con PMA no se ve modificada por la suplementación, de forma que los linfocitos de las mujeres porfíricas suplementadas con vitaminas C y E siguen produciendo mayores niveles de ROS que las mujeres control. De forma similar, la suplementación con vitaminas C y E potencia las actividades catalasa y glutatión reductasa eritrocitarias. Debido a que los eritrocitos no poseen la maquinaria necesaria para expresar genes ni sintetizar nueva proteína, los incrementos en la actividad enzimática pueden ser atribuidos a efectos directos sobre la proteína preexistente. Así pues, la suplementación durante 6 meses con dosis moderadas de vitaminas C y E otorga protección frente a la aparición de daño oxidativo en plasma e induce un incremento en la actividad de las defensas antioxidantes enzimáticas linfocitarias y eritrocitarias tanto en la población control como en mujeres afectadas de porfiria variegata.

Finalmente, en el último estudio de suplementación analizamos los efectos de una suplementación con la bebida anterior, rica en vitaminas C y E, pero también enriquecida con fitoestrógenos, sobre las respuesta antioxidante a una sesión de natación similar a la descrita con anterioridad. Determinados compuestos estrogénicos, como los fenilpropanoides presentes en el extracto de *Lippia citriodora*, presentan propiedades antioxidantes, antiproliferativas, antimetastáticas e inmunomoduladoras. Una sesión de natación de 30 minutos al 80% de la capacidad máxima induce un incremento de la actividad de los enzimas antioxidantes eritrocitarios relacionados con el metabolismo del glutatión, la glutatión peroxidasa y la glutatión reductasa, sólo en el grupo de nadadoras suplementadas con fitoestrógenos. En el linfocito, no observamos efectos de la suplementación sobre la actividad de los enzimas antioxidantes ni sobre la aparición de daño oxidativo en lípidos, proteínas y DNA. La actividad SOD linfocitaria aumenta tras el ejercicio en los dos grupos experimentales estudiados. De forma similar, el porcentaje de células con niveles altos de daño en el DNA aumenta en ambos grupos tras la sesión de natación. Se plantea así la posibilidad de que el consumo de nutrientes antioxidantes potencie las defensas antioxidantes endógenas de eritrocitos a través de mecanismos post-transcripcionales que pueden operar de forma inmediata como consecuencia de la realización de ejercicios de alta intensidad. Estas activaciones permitirían reducir de forma rápida un incremento de la producción de ROS. De persistir un cierto desequilibrio oxidativo, la sobreproducción de ROS podría llegar a inducir, en las células que disponen de núcleo, la expresión de genes antioxidantes que contribuirían a contrarrestar la producción de ROS. La acción post-transcripcional de los antioxidantes nutricionales depende de la concentración y capacidad antioxidante que presentan. Así, el

enriquecimiento adicional con fitoestrógenos produce un incremento de las actividades de enzimas antioxidantes eritrocitarias, que probablemente es adicional al que ya de por sí ha inducido el enriquecimiento con vitaminas E y C. Se ha postulado que determinados fitoestrógenos con actividad agonista/antagonista de hormonas estrogénicas podrían actuar a nivel de expresión de genes antioxidantes, procurando una mayor protección antioxidante como la que presentan las mujeres respecto de los hombres. Estos efectos no se han evidenciado en el caso de los fitoestrógenos presentes en el extracto de *Lippia citriodora*, ya que en ningún caso hemos observado mayores expresiones de genes antioxidantes en linfocitos asociados a su consumo. Sin embargo, sí que se han observado efectos muy marcados sobre los niveles circulantes de hormonas sexuales como consecuencia del consumo de estos fitoestrógenos. Al actuar los fitoestrógenos como agonistas/antagonistas de las hormonas estrogénicas, la ingesta de dosis relativamente altas de estos compuestos podría afectar a la propia producción de hormonas estrogénicas. Hemos observado una disminución en los niveles circulantes de las hormonas sexuales 17-β-estradiol, testosterona y testosterona libre. De esta forma, el verbascósido (fitoestrógeno principal presente en el extracto de *Lippia citriodora*) parece ejercer un efecto agonista del estradiol inhibiendo a la vez la síntesis endógena del propio estradiol, de forma similar a como éste regula sus síntesis por retroalimentación negativa.

Tomados en su conjunto, todos estos resultados evidencian la efectividad de los diferentes nutrientes antioxidantes a la hora de potenciar las defensas antioxidantes. La presencia de estos compuestos en niveles mayores en los individuos suplementados podría actuar directamente incrementando la detoxificación de las especies reactivas de oxígeno y sus productos de oxidación, pero también resulta evidente que estos compuestos son capaces de activar las propias defensas antioxidantes endógenas del organismo, especialmente los enzimas antioxidantes, cuando se ingieren en las dosis adecuadas. Los efectos de otros compuestos antioxidantes como los fitoestrógenos, que como se expone resultan potentes inhibidores de la síntesis de las hormonas estrogénicas endógenas, deben ser estudiados en mayor profundidad con el fin de determinar si la capacidad agonista/antagonista sobre las hormonas sexuales puede interferir en las capacidades reproductoras del individuo.

5. Modelos *in vitro* de inducción de adaptaciones al estrés oxidativo

Los resultados previos nos indican claramente que determinadas situaciones en las que la producción de, o exposición a, elevadas cantidades de especies

reactivas pueden inducir la activación de las defensas antioxidantes y la aparición de daño oxidativo. Sin embargo, al tratarse de modelos humanos y estudios *in vivo*, no es posible determinar cuáles son realmente los niveles de ROS a los las células se encuentran expuestas en estas situaciones de ejercicio o patologías como la porfiria. Por tanto, se hace evidente la necesidad de complementar estos resultados con otros que nos permitan establecer una relación clara entre los niveles de especies reactivas y los efectos producidos por éstas. Para ello diseñamos dos modelos experimentales de exposición a H_2O_2 detallados en el apartado de Planteamiento Experimental (apartado II.2.6). Con los resultados obtenidos a partir de estos experimentos queda patente que las células HL60 responden de forma marcadamente diferente a la exposición a H_2O_2 en función de los niveles y la persistencia de esta exposición. De todas las concentraciones probadas, sólo la exposición a H_2O_2 100 μM produce muerte celular, reduciendo la viabilidad al 80%, mientras que el resto de tratamientos, incluyendo las tres exposiciones sostenidas, no modifican la viabilidad celular. La exposición de las células HL60 a una dosis puntual de H_2O_2 10 μM induce la actividad de todos los enzimas antioxidantes (CAT, GRd, GPx y SOD), pero sin activar la expresión de sus genes. Esta activación de las defensas enzimáticas es efectiva a la hora de mantener la viabilidad celular, a pesar de que empieza a aparecer daño oxidativo a nivel de proteínas. La exposición a H_2O_2 100 μM también induce la actividad de los enzimas antioxidantes, pero a la vez se produce un descenso en la expresión génica, un incremento en la aparición de daño oxidativo y muerte celular, lo que parece sugerir que la activación de las defensas podría ser temporal e inefectiva a la hora de evitar el daño celular. Por el contrario, cuando las células se exponen a niveles bajos pero sostenidos de H_2O_2, éstas responden en una forma dosis-respuesta a la concentración de H_2O_2, potenciando tanto la expresión como la actividad de las defensas antioxidantes. A pesar de esta activación de las defensas, la exposición a concentraciones crecientes de H_2O_2 también induce una mayor capacidad celular de producción de especies reactivas y la aparición de daño oxidativo en proteínas y DNA. Teniendo en cuenta los resultados de ambos diseños, la exposición continua a H_2O_2 parece ser más apropiada a la hora de estudiar la respuesta antioxidante de las células HL60 al H_2O_2, ya que en condiciones fisiológicas las ROS se producen bajo mecanismos relativamente sostenidos más que de forma aislada. Cabe señalar que ninguna de los tratamientos con H_2O_2 estudiados se ha mostrado capaz de inducir la activación de las defensas antioxidantes sin provocar a la vez la aparición de daño oxidativo, lo que pone de manifiesto, una vez más y mostrándose de acuerdo con los resultados descritos en los apartados previos, que la activación de las defensas y la

aparición de daño oxidativo son fenómenos que ocurren de forma prácticamente paralela.

Los resultados presentados en esta tesis ponen de manifiesto la estrecha relación que se establece entre la activación de las defensas antioxidantes y la inducción de daño oxidativo ejercidas por las especies reactivas de oxígeno. Así pues, incluso las concentraciones más bajas de especies reactivas que hemos encontrado suficientes para activar las defensas conllevan también la aparición de daño oxidativo. Se han descrito diferentes mecanismos de activación génica de los enzimas antioxidantes en los que participan las ROS. Sin embargo, hemos aportado evidencias de que las ROS o sus productos de reacción también pueden potenciar de forma directa la actividad de los enzimas antioxidantes sin activar su expresión génica. Este otro mecanismo implica un tiempo de respuesta mucho menor para la célula que permite el hacer frente desde un principio a los incrementos de ROS. Si el estímulo es lo suficientemente persistente, entonces se produce la activación de la expresión con lo que se favorece una continuidad en la respuesta antioxidante. Los antioxidantes nutricionales ingeridos en concentraciones moderadas potencian los efectos que induce el ejercicio (y las ROS) sobre las defensas antioxidantes enzimáticas, sin modificar la expresión génica o los niveles de proteína, con una acción aparente sobre la proteína enzimática. El hecho de que elementos químicamente opuestos como son los prooxidantes y los antioxidantes tengan un efecto análogo o cooperativo sobre la actividad de los enzimas antioxidantes resulta paradójico. Los mecanismos moleculares a través de los cuales tanto las ROS como los antioxidantes inducen esta activación se desconocen por el momento, pero habría que considerar que muchos antioxidantes pueden actuar, en determinadas circunstancias, también como prooxidantes o que los antioxidantes proporcionan un efecto protector sobre la proteína enzimática antioxidante, o que el papel de activador del enzima sea el producto de la acción de las ROS sobre los antioxidantes suplementados. La ingesta de antioxidantes en las dosis utilizadas en este estudio no induce en ningún caso la aparición de daño oxidativo e incluso se muestra efectiva a la hora de disminuir los niveles de oxidación molecular, por lo que estos nutrientes parecen adecuados para potenciar la protección antioxidante y prevenir la aparición de daño oxidativo.

V. CONCLUSIONES

CONCLUSIONS

1. La exposición a oxígeno hiperbárico en ausencia de actividad física es suficiente para incrementar la producción linfocitaria de especies reactivas de oxígeno (ROS) y de activar las defensas antioxidantes en el linfocito. La combinación de hiperbaria y actividad física, propia del buceo con bombona, conduce a una situación de estrés oxidativo con daño muscular y la inducción de una respuesta inmunitaria de fase aguda. El plasma y los eritrocitos responden a esta situación incrementando sus defensas antioxidantes, y este incremento es suficiente para neutralizar la mayor producción de ROS y prevenir la aparición de daño oxidativo.

2. Una sesión de buceo con botella induce un incremento en los niveles de nitrito en plasma, eritrocitos y linfocitos. En los linfocitos este aumento en los niveles de nitrito parece ser consecuencia de una activación de la óxido nítrico sintasa inducible (iNOS). La activación de las defensas antioxidantes en plasma y en el eritrocito favorecen el incremento en los niveles de NO al aumentar la capacidad de eliminación de anión $O_2^{-}\cdot$ y por tanto reducir la posibilidad de formación de peroxinitrito.

3. La intensidad del ejercicio realizado es un factor importante que repercute en la respuesta del linfocito a la actividad física. La práctica de un ejercicio de alta intensidad genera un incremento en la producción linfocitaria de especies reactivas de oxígeno y en los niveles de peroxidación lipídica, a la vez que se activan las defensas antioxidantes a nivel transcripcional (hemooxigenasa 1) y post-transcripcional (glutatión peroxidasa). Otras proteínas con función antioxidante como son la UCP-3 y la Bcl-2 se ven negativamente reguladas en el linfocito tras la práctica de un ejercicio intenso, encontrándose una correlación inversa entre la expresión de UCP-3 y el daño oxidativo. El coactivador transcripcional PGC-1α y la sirtuina SIRT3 parecen ser efectores clave de la respuesta adaptativa del linfocito al ejercicio.

4. La práctica de ejercicio intenso induce en el neutrófilo una disminución de las defensas antioxidantes y, si la intensidad es suficiente, una explosión oxidativa y la aparición de daño oxidativo. La explosión oxidativa en el neutrófilo en respuesta a un ejercicio intenso coexiste con un incremento en la expresión de enzimas antioxidantes y una disminución en su actividad intracelular. Esto se explica, en el caso de la catalasa, por una salida controlada del enzima desde el neutrófilo hasta el medio extracelular.

5. La respuesta antioxidante al ejercicio difiere en función del género. Una sesión de actividad física intensa incrementa la producción de especies reactivas de oxígeno y de óxido nítrico, así como la expresión de iNOS y Mn-SOD, únicamente en chicos. La respuesta inflamatoria, así como el daño muscular y oxidativo, que se producen como consecuencia del ejercicio se ven parcialmente atenuadas en las chicas respecto de los chicos.

6. Las mujeres afectadas de porfiria variegata presentan una situación de estrés oxidativo crónico. Las defensas antioxidantes se encuentran activadas en eritrocitos de mujeres porfíricas, pero éstos también presentan mayores marcadores de estrés y daño oxidativo. Las actividades de los enzimas antioxidantes están disminuidas en linfocitos y neutrófilos en esta enfermedad, lo que en el caso del neutrófilo provoca la aparición de daño oxidativo. Las mujeres afectadas de porfiria variegata presentan además una alteración de las defensas antioxidantes mitocondriales en linfocitos. Esta menor protección antioxidante se ve acompañada de una mayor capacidad de producción de ROS por parte de la mitocondria en estas mujeres afectadas de porfiria. El resultado de la mayor producción de ROS y menores defensas antioxidantes en el linfocito de mujeres porfíricas es una mayor susceptibilidad al daño oxidativo en situaciones de estrés.

7. Las mujeres afectadas de porfiria variegata presentan un estado de leve inflamación crónica en comparación con mujeres sanas de la misma edad, caracterizado por la presencia de un mayor número de neutrófilos y monocitos circulantes, así como un incremento en lo niveles plasmáticos de proteína C reactiva y productos de peroxidación lipídica. El número incrementado de neutrófilos circulantes en mujeres porfíricas va acompañado de un mayor estado de activación de estos neutrófilos, evidenciado por la mayor capacidad de producción de ROS de éstos en respuesta a su estimulación inmunitaria.

8. La suplementación de la dieta con dosis moderadas de antioxidantes potencia la actividad de los enzimas antioxidantes. La ingesta de una bebida de almendra suplementada con vitamina C (150 mg/día) y vitamina E (50 mg/día) durante 6 meses potencia la actividad basal de los enzimas antioxidantes en linfocitos y eritrocitos, mediante mecanismos post-transcripcionales, a la vez que induce un descenso en los niveles de peroxidación lipídica en plasma en

mujeres afectadas de porfiria variegata y en mujeres sanas. La ingesta de la misma bebida, adicionalmente enriquecida con fitoestrógenos, durante 26 días potencia la activación de los enzimas antioxidantes eritrocitarios en respuesta a una sesión de natación en deportistas bien entrenados.

9. La suplementación de la dieta con un complejo multivitamínico y mineral enriquecido con coenzima Q (100 mg/día) durante 90 días en deportistas con una ingesta de vitaminas por encima de las CDR no modifica la adaptación de las células inmunitarias a un período de entrenamiento ni la respuesta adaptativa del neutrófilo a la práctica de una sesión de ejercicio intenso. Dicho suplemento se muestra, sin embargo, efectivo a la hora de evitar la aparición de daño oxidativo en lípidos y proteínas plasmáticos consecuencia de la actividad física.

10. El tratamiento de un cultivo de células HL60 con glucosa oxidasa y glucosa con una generación de H_2O_2 constante de entre 1 y 10 nM/s durante 1 hora supone un modelo experimental efectivo para generar una respuesta adaptativa al estrés oxidativo y simular el efecto de hormesis de las especies reactivas de oxígeno. La exposición sostenida de las células a estos niveles de H_2O_2 induce un incremento en la expresión génica y la actividad de las defensas antioxidantes enzimáticas, a la vez que activa la expresión del PGC-1α, un cofactor transcripcional que se encuentra a su vez implicado en la activación de numerosos genes. La activación de las defensas antioxidantes no previene la aparición inmediata de daño oxidativo, poniendo de manifiesto que la activación de las defensas y la aparición de daño oxidativo son fenómenos que ocurren de forma paralela.

11. El tratamiento de un cultivo de células HL60 con una dosis puntual de H_2O_2 10 y 100 μM potencia la actividad de los enzimas antioxidantes por mecanismos post-transcripcionales a la vez que disminuye su expresión génica, comprometiendo la respuesta celular a largo plazo. La exposición de las células HL60 a ambas dosis de H_2O_2 induce la aparición de daño oxidativo en proteínas, pero sólo se compromete la viabilidad celular cuando el H_2O_2 se suministra en concentraciones altas del orden de 100 μM.

1. The exposure to hyperbaric oxygen in the absence of physical activity is enough to increase the production of reactive oxygen species (ROS) and to activate the antioxidant defenses in lymphocytes. The combination of hyperbaria and physical activity present in scuba diving leads to a situation of oxidative stress with muscular damage and the induction of an acute phase immune response. Plasma and erythrocytes respond to this situation by increasing their antioxidant defenses, and this increase is enough to counteract the higher ROS production and prevent the apparition of oxidative damage.

2. A scuba diving session induces higher nitrite levels in plasma, erythrocytes and neutrophils. This increase in nitrite levels in lymphocytes can be attributed to the activation of inducible nitric oxide synthase (iNOS). The activation of the antioxidant defenses in plasma and erythrocytes enables the increase in nitrite levels by enhancing the $O_2^{\cdot-}$ scavenging, thus reducing the possibility of peroxinitrite formation.

3. The exercise intensity is an important factor affecting the lymphocyte response to physical activity. High-intensity exercise induces an increase in lymphocyte reactive oxygen species production and lipid peroxidation, while the antioxidant defenses are activated both at the transcriptional (heme oxygenase 1) and post-transcriptional (glutathione peroxidase) levels. Other proteins with known antioxidant function, such as UCP-3 and Bcl-2, are down-regulated in lymphocytes after intense exercise and a negative correlation between UCP-3 and oxidative damage is found. The transcriptional coactivator PGC-1α and sirtuin SIRT3 seem key effectors of this lymphocyte adaptative response to exercise.

4. Intense exercise induces a decrease in the antioxidant defenses of neutrophils, and an oxidative burst and oxidative damage appear when the intensity of exercise is high enough. The neutrophil oxidative burst in response to intense exercise coexists with an increase in the expression of antioxidant enzymes and a decrease in their activity. This is explained, at least for catalase, by a controlled output from neutrophil to the extracellular compartment.

5. The antioxidant response to exercise is influenced by gender. Physical activity increases reactive oxygen species and nitric oxide production, as well as iNOS and Mn-SOD expression, only in boys. The inflammatory response and the

muscular and oxidative damage in response to exercise are attenuated in girls when compared to boys.

6. Women affected by variegate porphyria present a situation of chronic oxidative stress. The antioxidant defenses are activated in erythrocytes of porphyric women, but these erythrocytes also present higher oxidative damage and stress markers. The antioxidant enzyme activities are decreased in lymphocytes and neutrophils from porphyric women and evidences of oxidative damage are found in neutrophils. Women affected by variegate porphyria also present impaired mitochondrial antioxidant defenses in lymphocytes. This minor antioxidant protection is accompanied by a higher mitochondrial ROS production capability in women affected by this disease. Higher ROS production and impaired antioxidant defenses in lymphocytes from variegate porphyria women result in increased susceptibility to oxidative damage in stress situations.

7. Women affected by variegate porphyria present a chronic inflammation condition when compared to control healthy women. This condition is characterized by higher neutrophil and monocyte counts and increases in the plasmatic levels of c-reactive protein and lipid peroxidation products. The increased neutrophil circulating counts in porphyric women is accompanied by a higher activation state of these neutrophils, as evidence by the increased ROS production after immune stimulation.

8. The diet supplementation with moderate dosages of antioxidants enhances the activity of antioxidant enzymes. The intake of an almond based beverage supplemented with vitamin C (150 mg/day) and vitamin E (50 mg/day) for 6 months enhances the basal activity of antioxidant enzymes in lymphocytes and erythrocytes through post-transcriptional mechanisms, while decreases the lipid peroxidation in plasma, both in women affected by variegate porphyria and control healthy women. The intake of the same beverage, but additionally enriched with phytoestrogens, for 26 days enhances the activation of the antioxidant enzymes in response to a swimming session in erythrocytes of well trained swimmers.

9. The diet supplementation with a multivitamin and mineral complex enriched with coenzyme Q (100 mg/day) for 90 days in sportsmen with a vitamin intake above the RDA does not modify the cellular adaptation to a training period nor the neutrophil adaptative response to intense exercise. This supplement is however effective in avoiding the apparition of oxidative damage in lipids and proteins from plasma as a result of exercise.

10. The treatment of a HL60 cell culture with glucose oxidase and glucose generating H_2O_2 in constant rates of 1 and 10 nM/s for 1 hour is an adequate experimental model to induce an adaptative response to oxidative stress and simulate the hormetic effect of ROS. The exposure of cells to a sustained production of 1 and 10 nM H_2O_2/s induces increased gene expression and activity of the antioxidant defenses and increases PGC-1α expression, a transcriptional cofactor involved in the activation of several genes. The activation of the antioxidant defenses can not avoid the apparition of oxidative damage, thus evidencing that the activation of defenses and the appearance of oxidative damage are processes that occur in a parallel course.

11. The treatment of a HL60 cell culture with a bolus of 10 and 100 μM H_2O_2 enhances the activity of antioxidant enzymes by post-transcriptional mechanisms while their gene expression is decreased, thus compromising the cellular response in a long term basis. The exposure of HL60 cells to both H_2O_2 dosages induces the apparition of oxidative damage in proteins, but the cell viability is only compromised when H_2O_2 is provided in concentrations as high as 100 μM.

VI. BIBLIOGRAFÍA

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