Free Radical Research, August 2007; 41(8): 874–883

informa
healthcare

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

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Accepted by Professor M. Jackson

(Received 15 January 2007; in revised form 3 April 2007)

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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ISSN 1071-5762 print/ISSN 1029-2470 online © 2007 Informa UK Ltd.
DOI: 10.1080/10715760701416459

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 (± 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%).

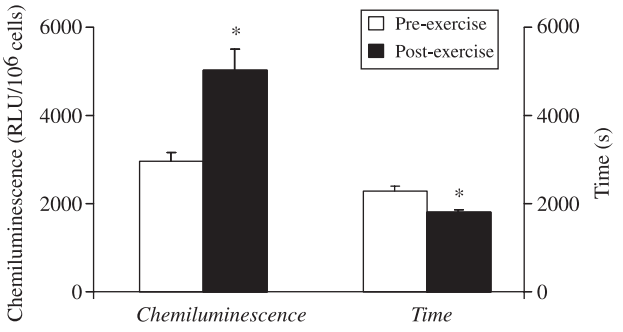

Figure 1. Effects of the cycling stage on neutrophil luminol chemiluminescence. Luminol chemiluminescence (RLU/10^6 cells) and the time of the maximum RLU (s) before and after the cycling stage. *t*-Student for paired data. (⋆) Indicates significant differences between pre and post-exercise, $n = 7$.

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

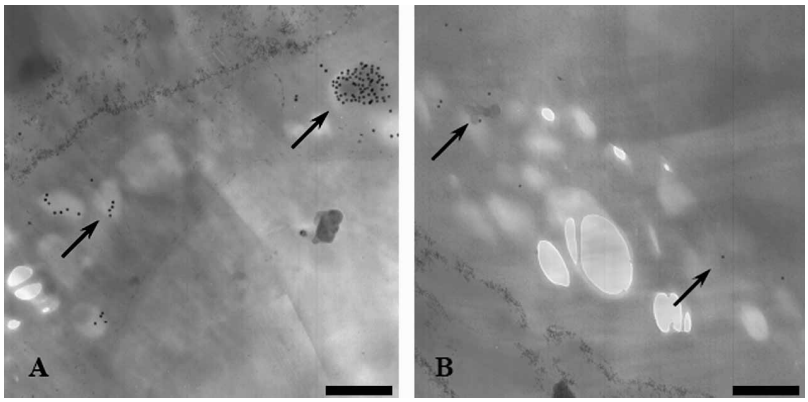

Figure 2. Effects of the cycling stage on neutrophil CAT localization. Immunogold localization of CAT (arrows). Representative electron micrograph of a region of neutrophil cytoplasm before (A) and after (B) exercise. Cells were incubated with cationized ferritin as a marker of plasma membrane. (Original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

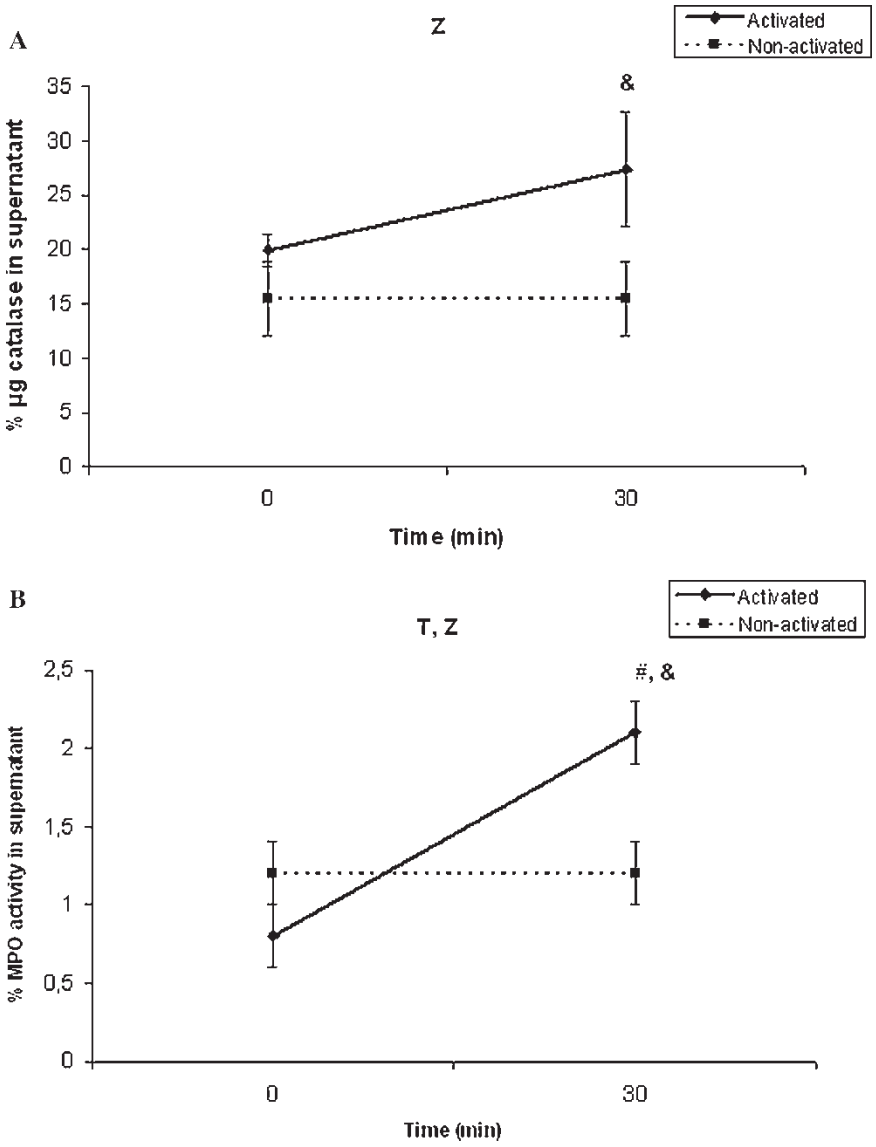

Figure 3. Percentage of CAT (A) and MPO activity (B) found in supernatant before and after a 30 min incubation of neutrophils with OZ. Statistical analysis: Two-way ANOVA. (*Z*) indicates significant effects of Zymosan activation. (*T*) indicates significant effects of time. ($T \times Z$) indicates 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.

Acknowledgements

This work has been granted by the Spanish Ministry of Health (Programme of Promotion of Biomedical Research and Health Sciences, Project PI021593), by the Spanish Ministry of Education and Science DEP-2005-00238-C04-02/EQUI to A Pons, and the FEDER funding. The authors are grateful to Dr F. Hierro and Ms M. Pocoví from the Servei Científic-tècnic of Universitat de les Illes Balears, and Ms E. Fuentespina for their technical assistance.

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