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Increased lymphocyte antioxidant defences in response to exhaustive exercise do not prevent oxidative damage

Pedro Tauler[a,*], Antoni Sureda[a], Nuria Cases[a], Antoni Aguiló[b], José A. Rodríguez-Marroyo[c], Gerardo Villa[c], Josep A. Tur[a,d], Antoni Pons[a,d]

[a]Departament de Biologia Fonamental i Ciències de la Salut, Universitat de les Illes Balears, Palma de Mallorca E-07122, Spain
[b]Departament d'Infermeria i Fisioteràpia, Universitat de les Illes Balears, Palma de Mallorca E-07122, Spain
[c]Facultad de Ciencias de la Actividad Física y del Deporte, Universidad de León, León E-24071, Spain
[d]Laboratori de Ciències de l'Activitat Física, Universitat de les Illes Balears, Palma de Mallorca E-07122, Spain

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Abstract

It has been reported that exercise induces oxidative stress and causes adaptations in antioxidant defences. The aim of this study was to determine the adaptations of lymphocytes to the oxidative stress induced by an exhaustive exercise. Nine voluntary male subjects participated in the study. The exercise was a cycling mountain stage (171.8 km), and the cyclists took a mean of 283 min to complete it. Blood samples were taken the morning of the cycling stage day, after overnight fasting, and 3 h after finishing the stage. We determined the blood glutathione redox status (GSSG/GSH), lymphocyte antioxidant enzyme activities and superoxide dismutase (SOD) levels; the plasma and lymphocyte vitamin E levels; the serum lactate dehydrogenase (LDH) and creatine kinase (CK) activities and urate levels; the plasma carotene and malonaldehyde (MDA) levels; and the lymphocyte carbonyl index. The cycling stage induced significant increases in blood-oxidized (glutathione/GSSG), plasma MDA and serum urate levels. The exercise also produced increases in CK and LDH serum activities. The mountain cycling stage induced significant increases in lymphocyte vitamin E levels, glutathione peroxidase and glutathione reductase activities as well as increased SOD activity and protein levels. The protein carbonyl levels increased significantly in lymphocytes after the stage. In conclusion, in spite of increasing antioxidant defences in response to the oxidative stress induced by the exhaustive exercise, lymphocyte oxidative damage was produced after the stage as demonstrated by the increased carbonyl index even in very well trained athletes.

Keywords: Exhaustive exercise; Oxidative stress; Lymphocytes; Antioxidants

1. Introduction

The benefits of regular moderate exercise on the well-being and the immune system are well known. However, these benefits are lost with exhaustion and lack of training. It has been reported that exhaustive exercise induces oxidative stress, which occurs when oxidant production overwhelms the antioxidant capacity [1–3]. Specific sources of reactive oxygen species (ROS) during exercise include leakage of electrons from the mitochondrial electron transport chain, xanthine oxidase reaction, haemoglobin oxidation and activated neutrophils [2,4]. The ROS has been shown to induce damage in all cellular macromolecules, such as lipids, proteins and DNA [3].

Leukocytosis, which includes both lymphopenia and neutrophilia, is well documented after exercise [5–8]. In fact, these events are included in the acute response of the immune system to exhaustive exercise. This response, which seems to be mediated by several cytokines, includes increases in activated complement components and neutrophil priming for oxidative activity, resulting in an inflammatory response.

It has been reported that antioxidant vitamins such as vitamin E and ascorbate are present in large concentrations in lymphocytes and that they play an important role in maintaining immune cell integrity and functionality [9,10]. Furthermore, human blood mononuclear cells express superoxide dismutase (SOD), catalase and glutathione (glutathione)

* Corresponding author. Tel.: +34 971 173171; fax: +34 971 173184.
E-mail address: pedro.tauler@uib.es (P. Tauler).

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peroxidase, and all these scavenging enzymes provide cellular defence against endogenous and exogenous ROS. Taking into account that exercise intensity could affect the immune response and that strenuous exercise induces a decrease in neutrophil antioxidant enzyme activities [11], it is important to know the effects of exhaustive exercise on the antioxidant defences, both endogenous and exogenous, of lymphocytes and their adaptations to the oxidative stress induced by this exercise. Some authors have suggested that the oxidative stress induced by exhaustive exercise could initiate apoptotic processes in lymphocytes, resulting in the lymphopenia observed after exhaustive exercise [12,13].

The aim of this study was to determine the response of lymphocyte antioxidant defences to an exhaustive exercise (a cycling stage) and the occurrence of oxidative stress during the cycling stage. The lymphocyte enzymatic antioxidant activities as well as the changes in lymphocyte vitamin E concentrations were analysed. Blood glutathione redox status, plasma MDA levels and the lymphocyte carbonyl index were determined in order to establish the occurrence of oxidative stress and cellular oxidative damage.

2. Materials and methods

2.1. Subjects and exercise

Nine voluntary male subjects participated in this study. They were all professional cyclists participating in the "Challenge Volta a Mallorca 2002", a 5-day competition for professional cyclists. Subjects were informed of the purpose of this study and the possible risks involved before giving their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki and was approved by the local ethics committee (Ethical Committee of University Hospital Son Dureta).

The exercise was a cycling mountain stage (171.8 km). This mountain stage was the third stage of the "Challenge Volta Ciclista a Mallorca 2002". The two previous stages were shorter (82.5 and 152 km) and did not include mountainous terrain. The cyclists' mean (±S.E.M.) age was 25.2±2.3 years, height 177±5 cm, weight 69.5±5.4 kg, body mass index 22.1±1.1 kg/m^2, 10.1±1.2 % fat and VO_2max 78.4±4.9 ml·kg^{-1}·min^{-1}. The mean±S.E.M. duration to complete this stage was 283±12 min.

2.2. Experimental procedure

Venous blood samples were taken from the antecubital vein with suitable vacutainers with EDTA as anticoagulant. Venous samples were taken following an overnight fast, before the race and 3 h after finishing the stage.

Blood samples were used to purify lymphocytes and to obtain plasma. Blood cells were quantified in an automatic flow cytometer analyser Technicon H*2 (Bayer) VCS system. We determined glutathione (GSH) and oxidized glutathione (GSSG) in blood. Vitamin E, antioxidant enzyme activities (catalase, glutathione peroxidase, glutathione reductase and SOD), Cu,Zn-SOD protein levels as well as carbonyl derivative levels were determined in lymphocytes. In plasma, we determined vitamin E, carotenes and MDA concentrations. We also determined creatine kinase (CK) and lactate dehydrogenase (LDH) activities and the urate levels in serum.

2.3. Lymphocyte and plasma purification

The lymphocyte fraction was purified following an adaptation of the method described by Boyum [14]. Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and centrifuged at 900×g, at 4°C for 30 min. The lymphocyte layer was carefully removed. The plasma and the Ficoll phases were 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.

Plasma was obtained after centrifugation (15 min, 1000×g, 4°C) of another blood sample obtained as above and was stored at −80°C until use.

2.4. Determination of urate and enzyme activities in serum

These determinations were made in an autoanalyser 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. The CK activity is proportional to the rate of increase in the concentration of NADPH [15]. Lactate dehydrogenase activity determination was based on the measurement of the conversion of pyruvate to L-lactate, by monitoring the oxidation of NADPH [16]. Both activities were monitored at 340 nm.

Uric acid was determined by an enzymatic method based on the specific uricase-catalysed oxidation of uric acid to allantoin and hydrogen peroxide [17].

2.5. Blood GSH/GSSG determination

GSH and GSSG were determined in blood by an adaptation of the method described by Winters et al. [18]. Blood was deproteinized with 30% trichloroacetic acid containing 2 mM EDTA. Then, two aliquots of 250 μl of the acidic supernatant were taken and neutralized with KOH/$NaHCO_3$ 2N. One aliquot was used to determine GSH. This aliquot was derivatized by the addition of NPM. Derivatization was completed within 5 min, and the fluorescent derivatives were then stabilized by acidification. At this point, the sample was injected into the HPLC system. In order to determine GSSG, 2-vinylpiridine was added to the second aliquot, and the mixture was incubated at room temperature for 60 min to block free GSH. Then GSSG was reduced enzymatically to GSH by the addition of NADPH and glutathione reductase in a very fast process. An aliquot was immediately taken out to measure GSH according to the procedure above.

The HPLC was a Shimadzu with a fluorescent detector operating at an excitation wavelength of 330 nm and an

Table 1
White blood cell counts and serum enzyme activities before and after the cycling stage

	Before	After
Leukocytes (10^3/μL)	5.96±0.25	14.6±1.5*
Lymphocytes (10^3/μL)	2.43±0.12	1.50±0.13*
CK (U/L)	219±36	342±77*
LDH (U/L)	306±14	418±19*

* Indicates significantly different values (Student's *t*-test paired data, $P<.05$).

emission wavelength of 380 nm. The column was a Waters Spherisorb 5 μm ODS2, 4.6×150 mm. Mobile phase consisted of 650:350 acetonitrile/H_2O acidified with 1 ml/L of acetic acid and 1 ml/L of *ortho*-phosphoric acid.

2.6. Plasma MDA determination

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

2.7. Lymphocyte protein carbonyl derivatives determination

Protein carbonyl derivatives were measured in lymphocytes by an adaptation of the method of Levine et al. [19]. After deproteinizing the samples with tricholoacetic 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 1000×*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 3000×*g* at 4°C to clarify the supernatant, and the absorbance was measured at 360 nm.

2.8. Vitamin E and carotene determination

Vitamin E was determined in plasma and lymphocytes. Carotenes were determined in plasma. The deep-frozen plasma or lymphocyte 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. Liposoluble vitamins and carotenoids were determined by HPLC in the *n*-hexane extract of plasma after drying under a nitrogen current and redissolving in methanol [20]. 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 was determined at 290 nm. Cryptoxanthin, β-carotene and lycopene were determined at 460 nm; lutein/zeaxanthin was determined at 450 nm.

Table 2
Changes in blood glutathione during the cycling stage

	Before	After
GSH (μmol/L)	1108±22	1234±74
GSSG (μmol/L)	23.9±0.8	27.7±1.0*
GSSG/GSH (%)	2.15±0.3	2.24±0.4

* Indicates significantly different values (Student's *t*-test paired data, $P<.05$).

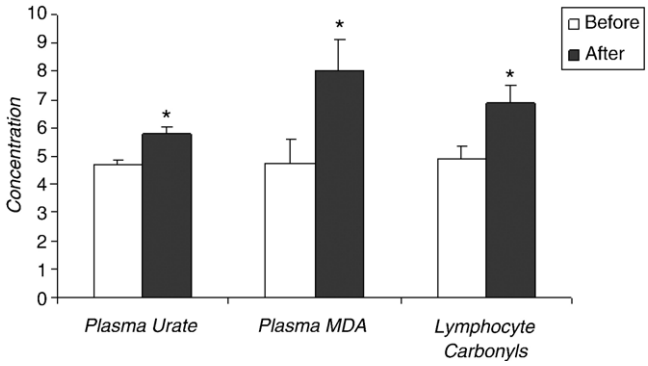

Fig. 1. Changes in plasmatic urate and MDA and in lymphocyte carbonyls. The asterisk indicates significantly different values (Student's *t*-test paired data, $P<.05$). Units: plasma urate (μg/L); plasma MDA (μmol/L); lymphocyte carbonyls (μmol/10^9 cells).

2.9. Lymphocyte antioxidant enzyme determination

We determined the activities of catalase, GSH peroxidase, GSH reductase and SOD in lymphocytes. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37°C.

Catalase activity was measured by the spectrophotometric method of Aebi [21] based on the decomposition of H_2O_2. Glutathione reductase activity was measured by a modification of the Goldberg and Spooner [22] spectrophotometric method. Glutathione peroxidase activity was measured using an adaptation of the spectrophotometric method of Flohe and Gunzler [23]. Superoxide dismutase activity was measured by an adaptation of the method of McCord and Fridovich [24].

2.10. ELISA determination of lymphocyte Cu,Zn-SOD levels

Superoxide dismutase levels were determined by ELISA using an anti-Cu,Zn-SOD polyclonal antibody (Sigma) that recognizes human Cu,Zn-SOD. We followed an adaptation of the method previously described [20].

Table 3
Changes in lymphocyte vitamin E and in plasma vitamin E and carotenes during the cycling stage

	Before	After
Lymphocytes		
Vitamin E (μM)	646±46	1008±74*
Plasma		
Vitamin E (μg/mL)	6.53±0.53	6.57±0.65
Lutein/zeaxanthin (μg/L)	341±53	377±49
Cryptoxanthin (μg/L)	239±26	210±27
Lycopene (μg/L)	90.4±14.2	88.2±20.5
β-Carotene (μg/L)	246±60	191±34

* Indicates significantly different values (Student's *t*-test paired data, $P<.05$).

2.11. Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS 9 for Windows). Results are expressed as means±S.E.M., and $P<.05$ was considered statistically significant. All the data were tested for their normal distribution. Student's *t*-test for paired data was used to determine the significance of the data.

3. Results

As shown in Table 1, significant increases in leukocyte counts and in serum CK and LDH activities were observed 3 h after finishing the cycling stage. However, the lymphocyte number decreased significantly after the stage.

Table 2 shows the changes in blood glutathione redox status during the cycling stage. Small, but not significant, increases were observed in the blood GSH levels and the GSSG/GSH ratio after the cycling stage. However, the cycling stage induced a significant increase, about 16%, in the blood GSSG concentration.

As indicators of the occurrence of oxidative stress and oxidative damage, changes in plasmatic urate and MDA levels and in lymphocyte carbonyl index are shown in Fig. 1. The cycling stage induced significant increases in the plasmatic levels of urate (23%) and MDA (69%). Furthermore, a 40% increase in the lymphocyte carbonyl index was observed after the cycling stage ($P<.05$).

The vitamin E concentrations in plasma and lymphocytes as well as the plasma carotenes before and after the cycling stage are presented in Table 3. No changes were found in the plasmatic vitamin E concentration. However, the lymphocyte vitamin E concentration increased significantly after the stage (56%). The cycling stage did not produce any changes in the plasmatic carotene levels.

Lymphocyte antioxidant enzyme activities and SOD protein levels before and after the cycling stage are presented in Table 4. No significant differences were found in lymphocyte catalase activity. However, significant increases were observed in glutathione peroxidase (87.6%), glutathione reductase (61.1%) and SOD (90.8%) activities. A significant increase was also observed in lymphocyte Cu,Zn-SOD protein levels (60.6%) after the stage.

Table 4
Changes in the lymphocyte antioxidant enzyme activities and SOD protein levels during the cycling stage

	Before	After
Catalase (K/10^9 cells)	23.6±3.3	28.5±3.9
Glutathione peroxidase (nkat/10^9 cells)	85.3±5.6	160±14*
Glutathione reductase (nkat/10^9 cells)	48.6±3.6	78.3±7.2*
Superoxide dismutase (pkat/10^9 cells)	13.1±2.1	25.0±2.6*
CuZn-SOD (pg/10^3 lymphocytes)	297±27	477±51*

* Indicates significantly different values (Student's *t*-test paired data, $P<.05$).

4. Discussion

There was no control trial in this study. However, the results obtained by ourselves and others in previous studies [5,10,25] allow us to assume that the changes reported are due to the exercise, and they are not associated with the time of the day the exercise has been performed and with possible influences of circadian variations in the measures [26–28].

Muscle damage, reticulocytosis and leukocytosis accompanied with lymphopenia are well-recognized phenomena following exhaustive exercise [29]. These were reflected in our study by the increased serum CK and LDH activities, a slight increase in the reticulocyte number (results not shown), the elevated leukocyte count and the decrease in lymphocyte number after the cycling stage.

Exercise-induced muscle damage has been widely reported following different eccentric exercises [30–32]. It has been suggested that this damage leads to a temporary loss of the exercising capacity of muscle for force production and has implications for increases in muscle soreness postexercise [33]. The higher levels of CK and LDH observed after the cycling stage may have resulted from a modest disruption of the muscle cell membrane, with the consequent leakage of proteins.

Our study showed, as indicators for oxidative stress during the cycling stage, higher plasmatic levels of MDA and urate, and increased GSSG blood levels. Lipid peroxidation causes a loss in fluidity and an increase in the permeability of membranes, resulting in loss of cytosolic proteins. It has been suggested that a high CK serum activity together with high levels of MDA, as in this work, could indicate that there is a relationship between the free radical attack induced by exercise and the protein leakage to plasma [2]. However, other studies did not find this relationship [34]. Several studies have reported increases in blood GSSG after exhaustive exercises [35,36]. In a previous study, we found a higher increase in the blood GSSG levels just after another cycling stage [37]. These increased levels were maintained 3 h after the stage, which supports the results obtained in the present study. Furthermore, in this work, we have found a small but not significant increase in the GSH blood content after exercise. This result is in agreement with others reporting increased GSH blood concentrations after a marathon run [8] and in highly trained cyclists after 2 h of cycling at 70% VO_2max [1]. It has been reported that reticulocytes and younger erythrocytes present higher levels of GSH [38], and this could account for the small increase in reticulocyte number found after the cycling stage. However, in a previous study the reticulocyte number had been normalized 1 week after the race, but the corresponding GSH level remained elevated [38]. Then, it is thought to be part of the self-protecting mechanism against oxidative stress activated during exercise [1]. Therefore, the increased blood GSH levels in the cyclists after the stage seem to result from mechanisms that were activated to compensate

for the ROS production during the exercise and the following short recovery. The increased GSH levels after the cycling stage could explain the maintenance of the GSSG/GSH ratio in spite of the increase in GSSG.

During intense exercise, the purine nucleotide system is extremely active [39]. The elimination of adenosine monophosphate (AMP) causes a build-up of hypoxanthine in skeletal muscle and plasma [39]. Although some may be converted back to AMP, hypoxanthine is also converted to uric acid via the xanthine dehydrogenase/xanthine oxidase (XDH/XO) system, increasing its plasmatic levels. We have shown a significant increase in uric acid after the cycling stage, indicating a significant increase in the purine metabolism. This result is consistent with previous findings where increases in plasma urate have been found after exhaustive exercises performed at different intensities [40]. Under conditions of oxidative stress, XDH is converted into XO, possibly through the oxidation of free sulfhydryl groups [40,41]. The XO-catalyzed reaction leads to the formation of reactive superoxide radicals and urate, which contribute, respectively, to increased oxidative stress and plasma urate levels. In fact, it has been suggested that XO is the main source of ROS during exercise and could be responsible for tissue damage associated with exhaustive exercise [41].

After confirming the occurrence of oxidative stress and oxidative damage during the cycling stage, we studied the response of the antioxidant systems to this oxidative stress. In agreement with previous findings [37], no changes were observed in the plasmatic concentrations of several carotenes after the stage. We have previously reported that the exercise-induced changes in carotenes are associated with changes in lipoprotein turnover, especially in LDL [42]. The lack of change in plasma carotene levels is associated with the maintenance of LDL observed after long-duration exercises such as a cycling stage [37]. Increases, decreases and no changes in plasmatic vitamin E have been observed after different bouts of exercise [37,43,44]. Plasmatic vitamin E changes could be a consequence of increased lipoprotein turnover or due to its mobilization along with triglycerides or via de novo synthesis of VLDL [42,45]. However, we show that plasmatic vitamin E did not change after this cycling stage. By contrast, lymphocyte vitamin E increased in response to exercise. These results are in agreement with the increase observed in lymphocyte vitamin E just after a half marathon, with the levels maintained high after 3 h of recovery [10]. It seems that vitamin E lymphocyte uptake is activated by oxidative stress in order to protect the cell against the action of the ROS. However, the mechanisms underlying this vitamin E uptake in lymphocytes are unknown in spite of reports of expression of the protein α-tocopherol transfer protein (α-TTP) in leukocytes [46].

The increased vitamin E content in lymphocytes could be part of the cell response to increased oxidative stress induced by the cycling stage because the antioxidant enzyme activities also increased in lymphocytes after the stage. Previous studies reported that the expression of the antioxidant enzymes in the immune cells is induced and regulated by ROS and cytokines, along with other factors [47]. Recently, it has been reported that contracting skeletal muscle generates ROS that can induce changes in gene expression or cell damage depending upon the pattern of production and the endogenous protective systems [48]. Then, the skeletal muscle could use contraction-induced ROS as signals to induce adaptive responses including maintenance of oxidant homeostasis and prevention of oxidative damage [48]. In fact, it has been shown that the enzymatic adaptations to oxidative stress during exhaustive exercise induced by ROS were abolished when the ROS production was prevented [49,50]. In agreement with the role of ROS as inducers of enzymatic adaptations to oxidative stress, we show in this work that a significant increase in enzymatic protein levels of lymphocyte Cu,Zn-SOD is produced after the cycling stage. Then, we can conclude that increased SOD activity was due to the increase in expression induced by oxidative stress. In a similar way, increases in lymphocyte heme oxygenase-1 (HO-1) mRNA expression after different exercises [25,51], but also in HO-1 protein levels 2 h after exercise, were found [25]. However, the adaptive response of antioxidant defences, both endogenous and exogenous, did not prevent the oxidative damage in lymphocytes as indicated by the increased carbonyl index after the stage. We can hypothesize that the pattern of ROS production simultaneously induced an adaptive response and oxidative damage in the lymphocyte.

Although the lymphocyte population is dynamic, we suggest that the changes reported above are due to cellular adaptations to the oxidative stress, and they are not produced by migration of cells out of the circulation and the arrival of new cells into the circulation. In summary, a mountain cycling stage as an exhaustive exercise induces oxidative stress and muscular damage. This oxidative stress induces antioxidant adaptations in lymphocytes in order to avoid oxidative damage via increases in both enzymatic activities and expression, and nonenzymatic concentrations such as increased vitamin E content. However, these adaptations did not prevent the occurrence of oxidative damage in lymphocytes even in very well trained athletes such as the ones who participated in this study.

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