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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.

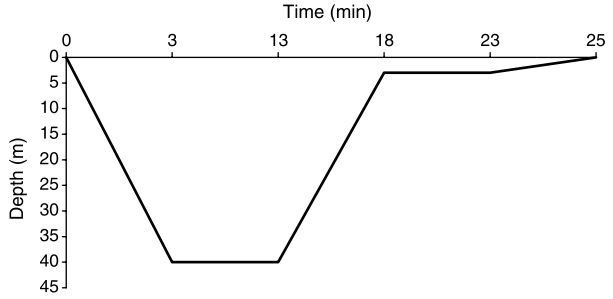

Figure 1. Schedule of the scuba diving session profile.

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.

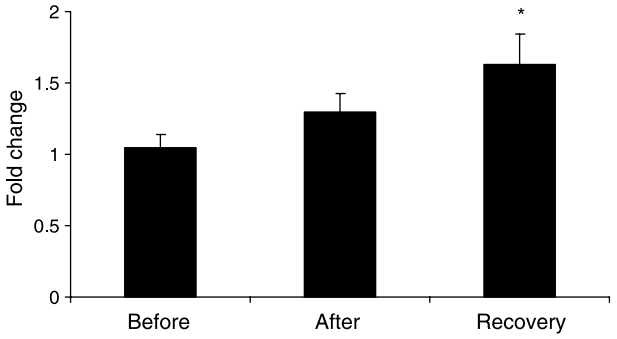

Figure 2. Changes in lymphocyte HO-1 gene expression after a scuba diving session. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. *t*-student for paired data; (*) indicates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

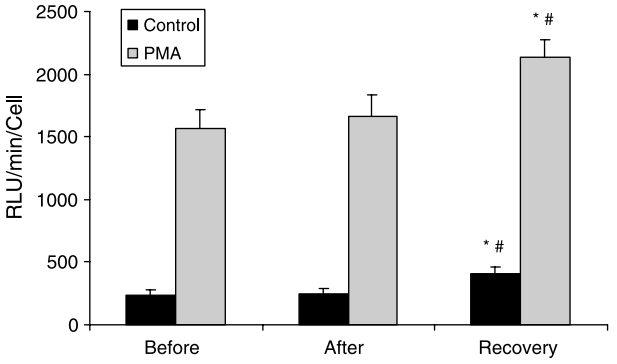

Figure 3. Effects of a scuba diving session on lymphocyte H_2O_2 production. H_2O_2 production in control and in PMA activated lymphocytes, before and after a HBO session. One-way ANOVA (⋆) significant differences with respect to the before values. (#) Significant differences between after and recovery,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

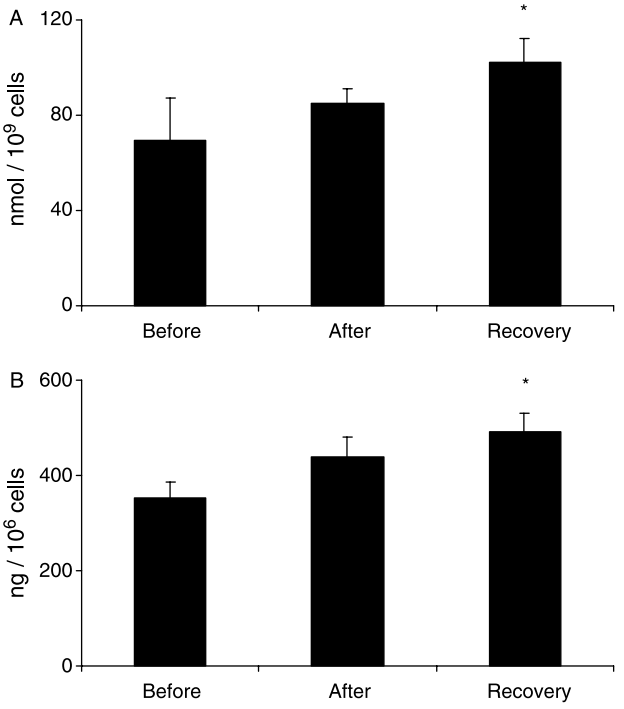

Figure 4. Changes in lymphocyte nitrite (A) and iNOS protein levels (B) in response to a scuba diving session. One-way ANOVA (⋆) significant differences between before and after. (#) Significant differences between before and recovery, $p < 0.05$. Mean ± SEM.

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).

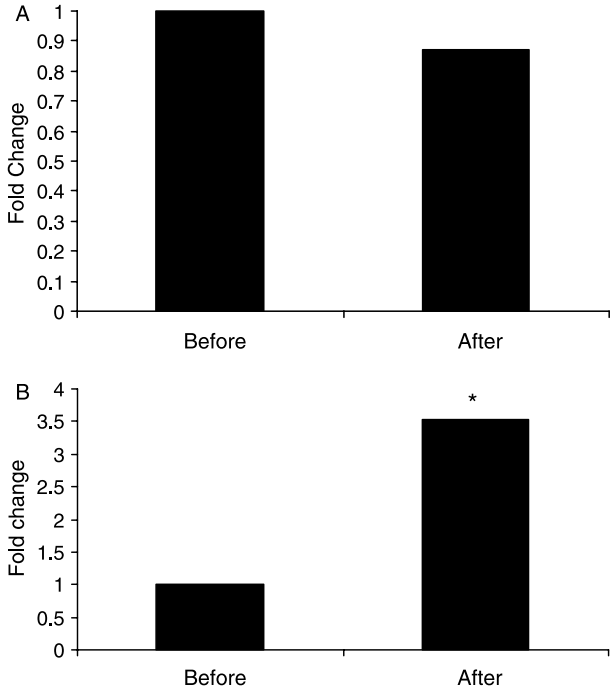

Figure 5. Effects of an HBO exposure on lymphocyte GPx (A) and HO-1 (B) gene expression. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. *t*-student for paired data; (*)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

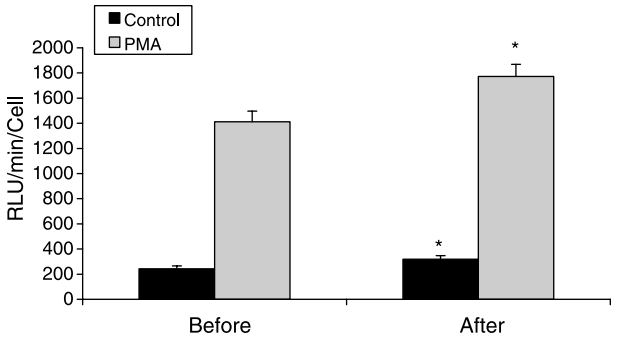

Figure 6. Effects of an HBO treatment on lymphocyte H_2O_2 production. H_2O_2 production in control and in PMA activated lymphocytes, before and after a HBO session. One-way ANOVA (*) significant differences with respect to the before values, $p < 0.05$. Mean ± SEM.

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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