Scuba Diving Increases Erythrocyte and Plasma Antioxidant Defenses and Spares NO without Oxidative Damage

AQ1 ANTONI SUREDA[1], MIGUEL D. FERRER[1], JOAN M. BATLE[2], PEDRO TAULER[1], JOSEP A. TUR[1], and ANTONI PONS[1]

[1]*Laboratory of Physical Activity Sciences, University of the Balearic Islands, Palma de Mallorca, Balearic Islands, SPAIN; and* [2]*MEDISUB, Alcúdia, Balearic Islands, SPAIN*

ABSTRACT

SUREDA, A., M. D. FERRER, J. M. BATLE, P. TAULER, J. A. TUR and A. PONS. Scuba Diving Increases Erythrocyte and Plasma Antioxidant Defenses and Spares NO without Oxidative Damage. *Med. Sci. Sports Exerc.*, Vol. 41, No. 6, pp. 000–000, 2009. **Purpose**: The aim of the present work was to study the effects of a single scuba diving immersion to high depth on erythrocyte and plasma antioxidant defenses, on erythrocyte cellular damage, and on nitric oxide (NO) production. **Methods**: Seven male preprofessional divers performed an immersion at a depth of 40 m for a total time of 25 min. Blood samples were obtained before the diving session after overnight fasting, immediately after diving, and 3 h after the diving session was finished. Erythrocytes and plasma fractions were purified. **Results**: No significant differences were found in circulating erythrocytes, bilirubin, and hemoglobin concentration attributed to diving. Hematocrit levels were reduced after diving because of the reduction of erythrocyte size that was maintained after 3 h of recovery at the surface. Leukocyte counts significantly increased at recovery (38 ± 4%). In erythrocytes, glutathione peroxidase activity significantly increased (18 ± 4%) at recovery. A rise in plasma catalase activity (38 ± 6%) immediately occurred after diving, returning to basal values after recovery. Plasma superoxide dismutase activity significantly increased (58 ± 7%) during recovery. Markers of oxidative damage in both erythrocytes and plasma such as malondialdehyde and protein carbonyl derivates remained unchanged after diving. Nitrite levels significantly rose in plasma and erythrocytes (85 ± 8% and 52 ± 6%, respectively) at recovery. **Conclusion**: Scuba diving session induced an antioxidant response in plasma and erythrocytes without the appearance of cellular damage and an increase in NO, which can be related with its vasodilator role. **Key Words:** OXIDATIVE STRESS, ANTIOXIDANTS, HYPERBARIC OXYGEN, NITRIC OXIDE, ERYTHROCYTES

Exposure to hyperbaric oxygen (HBO) favorably leads to an increase of dissolved oxygen in the blood, and it has been successfully used as an adjunctive therapy for several ischemia/reperfusion injuries, including decompression sickness, acute carbon monoxide intoxication, or impaired wound healing (16,45). HBO can relieve tissue hypoxia, restore the oxygen necessary for normal oxidative metabolism, and stimulate repair and angiogenesis (40,51). However, it has been demonstrated that HBO also leads to an increased reactive oxygen species (ROS) formation that can cause cellular damage (22,30). The cellular response to HBO-induced oxidative stress has been mainly investigated in animal models (15,23), and there are few studies performed in humans (5,12).

Altogether, erythrocytes and plasma are the most susceptible blood fractions to suffer from lipid oxidative damage induced by ROS (37). Erythrocytes are susceptible to oxidative damage because of the high polyunsaturated free fatty acid content of their membranes and the high cellular concentrations of oxygen and hemoglobin, a potentially powerful promoter of oxidative processes, and because they are unable to repair damaged components by resynthesis (10). However, erythrocytes contain an elaborate antioxidant defense system that involves antioxidant enzymes that eliminate ROS (37). Antioxidants in both hydrophilic and lipophilic compartments of plasma are actively involved as a defense system against ROS, which are continuously generated (50).

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

Address for correspondence: Antoni Pons, Laboratori de Ciències de l'Activitat Física, Universitat de les Illes Balears, Ctra. Valldemossa, km 7.5, E-07122 - Palma de Mallorca, Balears, Spain; E-mail: antonipons@uib.es.

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diving requires a decompression before return to the surface to eliminate the excess of gas accumulated during the stay under water. Decompression sickness is related to the vasoconstriction together with the generation of nitrogen bubbles. Venous gas bubbles are regularly observed in divers, and high amounts of bubbles are linked to an increased incidence of decompression sickness (14). Dujic et al. (13) evidenced that exercise before diving and during decompression reduces the number of gas bubbles, probably through a nitric oxide (NO) mechanism. It has been shown that NO decreases the amount of vascular bubbles produced by decompression in rats (49), whereas the NO-inhibitor L-NAME increases the amount of circulating bubbles (48). NO could induce the peripherical vasodilatation decreasing the generation of gas bubbles in the main vessels and reducing the pernicious effects of the decompressive sickness (49). NO, mainly synthesized by endothelial cells, diffuses to the lumen where it is either oxidized by oxygen, resulting in the formation of nitrite, or taken up by erythrocytes (11). Nitrite is the main end-product of NO metabolism, and it is a relatively stable molecule under intracellular reducing conditions and has recently been pointed out as a storage pool for NO synthesis in erythrocytes (19). NO availability is associated to the superoxide anion production because both molecules rapidly react to form peroxynitrite, a harmful oxidizing agent (33).

In a previous study, we evidenced that the hyperoxia associated to scuba diving leads to a condition of oxidative stress with increased serum markers of cellular damage, namely, creatine kinase (CK) and lactate dehydrogenase (LDH), although it did not occur after HBO therapy (17). We hypothesize that scuba diving (hyperoxia and HBO) induces responses in erythrocytes and plasma to maintain the redox balance, to prevent oxidative damage, and to facilitate NO function. The aim of the present work was to study the effects of a single scuba diving immersion to a high depth on the erythrocytes and plasma antioxidant system, on the oxidative damage, and on the NO synthesis.

MATERIALS AND METHODS

Subjects and study design. Seven male preprofessional divers (34.5 ± 3.6 yr; body mass index (BMI) = 23.1 ± 0.6 $kg \cdot m^{-2}$) volunteered to take part in this study. The subjects were nonsmoker scuba diving learners, and they did not take any antioxidant dietary supplement or any routine medication for 1 month before the study. All participants, at the time of the study, had a valid medical certificate for diving. The protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the CAIB (Palma de Mallorca, Balearic Islands, Spain). Subjects were informed of the purpose and demands of the study before giving their written consent to participate. Previously to the immersion, all sportsmen passed a physical and medical test in the laboratory including an electrocardiographic evaluation, a maximal test using a cycloergometer with heart rate and lactacidemia controls, and a blood test (hematological and serum biochemical parameters) to ensure a good performance to prac tice this sport. All analyzed subjects passed the medical test. Subjects were instructed to refrain from strenuous exercise and to ingest their habitual diet without any supplement during the 48 h preceding each laboratory test. Divers performed an immersion at a maximum depth of 40 m for 25 min during which they breathed atmospheric air. The return to the surface was with a decompression of 5 min at a depth of 3 m.

Experimental procedure. Venous blood samples were obtained from the antecubital vein of divers in suitable vacutainers. Venous blood samples were obtained before the diving session after overnight fasting (basal sample), immediately after diving, and 3 h after finishing the diving session. Plasma and erythrocytes were purified. Antioxidant enzyme activities, markers of oxidative damage, and nitrite levels were measured in both plasma and erythrocytes. Hematological parameters such as erythrocyte and leukocyte number and hematocrit and hemoglobin concentration were determined in an automatic flow cytometer analyzer Technicon H2 (Bayer) VCS system. Bilirubin concentration was also determined in serum. Hematological parameters and bilirubin concentration were analyzed before the first hour after extraction. AQ2

Bilirubin determination. Total bilirubin is defined as the amount of bile pigment in serum or plasma that reacts with diazosulphanilic acid into an acid pH to produce azobilirubin (28). Unconjugated bilirubin is solubilized by an accelerator such as caffeine, organic solvents, or surfactants. The end point absorbance, measured at 548 nm, is directly proportional to the concentration of total bilirubin.

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

Antioxidant enzyme activities. Catalase (CAT) activity was measured by the spectrophotometric method of Aebi (1) on the basis of the decomposition of H_2O_2. Glutathione reductase (GR) activity was measured by a modification of the Goldberg and Spooner (21) spectrophotometric method. This assay required oxidized glutathione as the substrate. Glutathione peroxidase (GPx) activity was measured using an adaptation of the spectrophotometric method of Flohe and Gunzler (18). This assay required H_2O_2 and NADPH as substrates and GR as enzyme indicator. The

capability to detoxify hydroperoxides (GPer) was carried out in the same way as for GPx, but the substrate was cumene hydroperoxide. Superoxide dismutase (SOD) activity was measured by an adaptation of the method of McCord and Fridovich (26). 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. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37°C.

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

MDA–protein adducts and protein carbonyls were determined by an enzyme immunoassay (Cell Biolabs, Inc.). Briefly, standards or protein samples (10 $\mu g \cdot mL^{-1}$) were adsorbed onto a 96-well plate and incubated overnight at 4°C. MDA–protein adducts were probed with an anti-MDA antibody, followed by an HRP-conjugated secondary antibody. The protein carbonyls present in the sample or standard were derivatized with dinitrophenylhydrazine (DNPH) to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP-conjugated secondary antibody. Quantification was performed by comparing unknown samples with a standard curve of known concentration.

Nitrite determination. Nitrite levels were determined in lymphocytes by the acidic Griess reaction using a spectrophotometric method. Lysed cells were deproteinised with acetone and kept at −20°C overnight. 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 nitrite standard solutions (100 μL) in duplicate. Fifty microliters of sulfanilamide (2% w/v) in 5% HCl was added to each well, and 50 μL of *N*-(1-naphthyl)-ethylenediamine (0.1% w/v) in water was later added. The absorbance at 540 nm was measured after an incubation of 30 min.

Statistical analysis. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS 14.0 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 repeated-measures ANOVA.

TABLE 1. Hematological and biochemical parameters.

	Before	After	Recovery
Erythrocytes ($10^6 \cdot \mu L^{-1}$)	5.02 ± 0.06	4.85 ± 0.08	4.88 ± 0.09
Hemoglobin ($g \cdot dL^{-1}$)	15.1 ± 0.1	14.5 ± 0.2	14.7 ± 0.2
Hematocrit (%)	44.3 ± 0.5	42.0 ± 0.6*	42.0 ± 0.7*
VCM (fL)	88.4 ± 0.7	86.7 ± 0.5*	86.1 ± 0.6*
HCM (pg)	30.2 ± 0.2	30.0 ± 0.3	30.3 ± 0.3
CHCM ($g \cdot dL^{-1}$)	34.2 ± 0.2	34.6 ± 0.2	35.1 ± 0.2*
RDW (%)	13.3 ± 0.1	13.5 ± 0.1	13.5 ± 0.1
Bilirubin ($mg \cdot dL^{-1}$)	1.03 ± 0.08	1.02 ± 0.09	1.06 ± 0.08
Leukocytes ($10^3 \cdot \mu L^{-1}$)	6.74 ± 0.34	7.08 ± 0.35	9.32 ± 0.6*†

Hematological and biochemical parameters before and after scuba diving and after 3 h of recovery. One-way ANOVA. Values represent mean ± SEM.
* Significant differences respect to before values.
† Significant differences between after and recovery, $P < 0.05$.

TABLE 2. Erythrocyte and plasma antioxidant enzyme activities.

	Before	After	Recovery
Erythrocytes			
CAT (kat per 10^9 cells)	3.69 ± 0.19	3.25 ± 0.21	3.29 ± 0.25
GPx (nkat per 10^9 cells)	11.5 ± 0.5	10.7 ± 0.4	11.1 ± 0.5
GPer (nkat per 10^9 cells)	5.68 ± 0.25	6.28 ± 0.26	6.69 ± 0.43*
GR (nkat per 10^9 cells)	4.96 ± 0.30	5.04 ± 0.29	5.18 ± 0.36
SOD (pkat per 10^6 cells)	0.87 ± 0.05	0.88 ± 0.04	0.91 ± 0.03
Plasma			
CAT ($kat \cdot L^{-1}$ plasma)	200 ± 18	276 ± 23*	232 ± 19
GPx ($nkat \cdot L^{-1}$ plasma)	494 ± 16	541 ± 40	553 ± 45
SOD ($pkat \cdot L^{-1}$ plasma)	48.9 ± 6.4	72.1 ± 9.5	77.3 ± 9.4*

Erythrocyte and plasma antioxidant enzymes before and after scuba diving and after 3 h of recovery. One-way ANOVA. Values represent mean ± SEM.
* Significant differences respect to before values, $P < 0.05$.

RESULTS

Table 1 presents the hematological data obtained in the seven divers at each experimental time. There were no significant differences in circulating erythrocytes, bilirubin, blood hemoglobin concentration, mean hemoglobin content of erythrocytes (HCM), and the distribution of erythrocyte sizes (RDW). Hematocrit levels and the mean corpuscular volume of erythrocytes (VCM) were reduced immediately after the dive, and this reduction was maintained after 3 h of recovery and the hemoglobin erythrocyte concentration (CHCM) significantly increased during recovery. Leukocyte counts significantly increased at recovery 38 ± 4% ($P < 0.05$).

A short dive session of 25 min at a 40-m depth was enough to induce erythrocyte and plasma antioxidant adaptations. Erythrocyte and plasma antioxidant enzyme activities are shown in Table 2. Erythrocyte GPer activity significantly increased 18 ± 4% ($P < 0.05$) after 3 h of recovery, whereas the other antioxidant enzyme activities maintained the basal values. There was an increase in plasma CAT activity immediately after diving (38 ± 6%; $P < 0.05$). CAT activity returned to the basal values after recovery. Plasma SOD activity significantly increased during recovery 58 ± 7% ($P < 0.05$).

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

TABLE 3. Oxidative damage markers.

	Before	After	Recovery
MDA			
Plasma ($\mu mol \cdot L^{-1}$ plasma)	11.8 ± 1.1	10.0 ± 0.9	12.5 ± 0.9
Erythrocytes (nmol per 10^9 cells)	13.9 ± 0.7	13.9 ± 0.8	13.2 ± 0.8
Protein carbonyl derivates			
Plasma ($nmol \cdot mg^{-1}$ protein)	0.66 ± 0.06	0.67 ± 0.05	0.69 ± 0.05
Erythrocytes ($nmol \cdot mg^{-1}$ protein)	18.7 ± 1.9	18.9 ± 2.0	19.2 ± 1.9
MDA–protein adducts			
Plasma ($nmol \cdot mg^{-1}$ protein)	0.76 ± 0.06	0.77 ± 0.06	0.73 ± 0.08
Erythrocytes ($nmol \cdot mg^{-1}$ protein)	2.90 ± 0.21	2.97 ± 0.25	2.99 ± 2.82

MDA was measured with a colorimetric assay, and protein carbonyl derivates and MDA–protein adducts were measured with immunological techniques in plasma and erythrocytes before and after scuba diving and after 3 h of recovery. No significant differences were evidenced, one-way ANOVA. Values represent mean ± SEM.

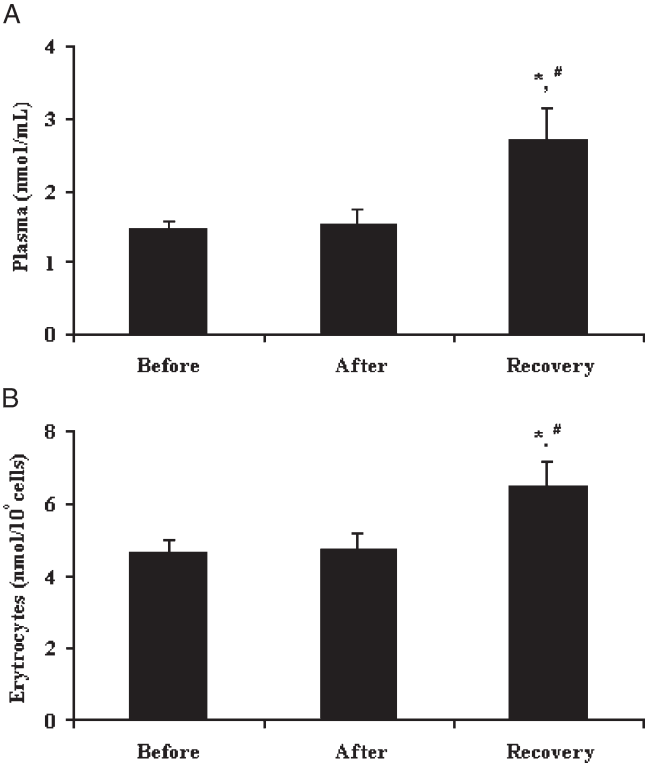

FIGURE 1—Plasma and erythrocyte nitrite concentration. Nitrite levels before and after scuba diving and after 3 h of recovery. One-way ANOVA. *Significant differences respect to before values. #Significant differences between after and recovery, $P < 0.05$. Values represent mean ± SEM.

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

DISCUSSION

Plasma and erythrocyte antioxidant defenses and NO production changed as result of a short immersion for 25 min at a maximum depth of 40 m. Scuba diving submits the organism to hyperbaric hyperoxia, which could increase ROS generation leading to tissue damage. The physiological stress of scuba diving in comparison with dry chambers is greater because of additional factors such us immersion, exercise, and cold water. The selected sample and the stressor condition (depth) could limit the generalization of the study. However, an open sea diving at 40 m requires a very good health and physical status. Sedentary or nontrained people are not allowed to perform immersions to high depths. The training status of divers will probably result in a better tolerance to the stress situation because it has been well established that regular training increases antioxidant basal situation in athletes (2,3). It was described that diving animals presented, in general, higher antioxidant status compared with nondiving mammals (45). This higher antioxidant status of diving animal allows to prevent tissue exposure to high oxygen levels during reoxygenation and to avoid the oxidative stress condition related to increased ROS generation (47). It was also known that endurance training in animals or good physical conditioning in divers reduce the venous bubble formation and decrease the incidence of decompression sickness (7,9,31). Scuba diving at 40 m for 25 min was enough to provoke a leukocyte mobilization suggesting an inflammation-like response. This rise represents the rapid recruitment of leukocytes from marginated pools, like it occurs after infection or after exercise (8,38,39). Other study evidenced an increase in the absolute number of granulocytes after a single dive to 54 m for 20 min of bottom time (20). The scuba diving session significantly reduced the hematocrit value in agreement with previous studies (44). The hematocrit and VCM reduction as a whole with the maintenance of the number of erythrocytes, blood hemoglobin concentration, and serum bilirubin levels indicated that erythrocytes changed their size but without apparent hemolysis. The decrease in erythrocyte volume induced by the high pressure during diving concentrated the hemoglobin into the cells as evidenced by the increased CHCM values.

The lipid peroxidation process of membranes has been pointed as one of the primary events in oxidative cellular damage and has been shown to be associated with fine structure disturbance and subsequent function loss of biological membranes (4). Because mature human erythrocytes do not have nucleus or other organelles, the plasma membrane in these cells is the critical target (37). Erythrocytes are exposed to ROS that are constantly generated from both internal and external sources even under normal conditions, and they may be targeted for oxidative damage during the hyperbaric hyperoxia induced by scuba diving. Although a direct relation cause–effect has to be demonstrated, the higher GPer activity after diving could be indicative of increased levels of lipid hydroperoxides. In fact, we previously evidenced that GPer increased in erythrocytes during the recovery of a cycling stage (41). Erythrocytes cannot synthesize proteins, so that the increase in the maximal activities of the enzymes could be attributed to covalent modifications of proteins or to other protein interactions (41). The increased activity of GPer could also be a result of the activation of the enzyme by ROS or by its own substrate, the lipid hydroperoxide. However, markers of cellular damage were unchanged in erythrocytes indicating that the cellular antioxidant response is enough to avoid the ROS-induced damage. In previous results, we evidenced a significant increase in MDA and carbonyl groups as measured with colorimetric assays after a cycling mountain stage (42). However, to avoid the possibility of a false-negative of the colorimetric assays to determine MDA and protein carbonyls, more sensitive immunological techniques were used in the present study.

Nitrite is a relatively stable end-product of NO metabolism, and it has been also pointed out as a storage pool for NO synthesis from erythrocytes that produce vasodilatation

into the hypoxic tissues (19). NO metabolism is influenced by the presence of superoxide anion, which reacts with NO to delivery peroxynitrite. Then, an increase in superoxide anion could induce a decreased availability of NO and could produce vasoconstriction. It was evidenced that application of short-acting NO donor (nitroglycerine before diving) reduced the number of bubbles observed in the right side of the heart of divers (13). During the initial stage of hyperbaric oxygenation, there is an increase in superoxide anion production, resulting in NO inactivation and, thus, vasoconstriction (52). This hyperoxia-derived vasoconstriction may act as a trigger for an increased NO production, which reverses vasoconstriction (27). We evidenced an increase in NO production 3 h after scuba diving in both plasma and erythrocytes. The return to normobaria and normoxia requires vascular vasodilatation, and consequently, the NO production is reinforced. We have previously evidenced that vitamin C diet supplementation increased the erythrocyte NO production induced by hypoxia/reoxygenation (38). Then, the erythrocyte oxidative status could alter the NO production by the erythrocytes.

The physiological exposure to scuba diving will lead to an increase in antioxidant enzyme activities, which could result in the preservation of NO function. It was evidenced that when antioxidant power was increased (after 4 wk of antioxidant supplementation), the negative effects of a scuba diving on endothelial function were attenuated (29). Previous studies evidenced an increase in the total antioxidant capacity measured in plasma after breathing oxygen under pressure (25). In our study, CAT and SOD activities significantly increased after scuba diving, enhancing the plasma antioxidant status. This rise in antioxidant enzymes could be important to avoid oxidative damage induced by ROS overproduction and to allow the vasodilatory function of NO. It has been shown that 1 h after the dive, microbubbles could be detected in the heart chambers of divers (6). The increase in NO during recovery could be important to avoid the potential deleterious effects of air bubbles and to prevent the decompression sickness. In fact, it has been described that hypertensive patients have a reduction of the extracellular SOD activity (53). The increase of SOD in plasma could be important to avoid the reaction between the superoxide anion and the NO and, consequently, to reduce the formation of peroxynitrite. Rousseau et al. (32) evidenced an increase in GP activity and a decrease in glutathione after a submaximal apnea dynamic diving to buffer against the increase of ROS. It also described an increase in SOD activity in human patients with rheumatoid arthritis under HBO therapy (24). The increased activity of plasma SOD could produce an increased production of hydrogen peroxide in plasma. This point is also counteracted by a similar rise of plasma CAT. The increased activities of these enzymes could be attributed to an increase of their plasma levels in addition to the mechanisms that activate the antioxidant enzymes in erythrocytes. We previously pointed out the possibility to secrete CAT from neutrophils during intense exercise as a part of the acute phase immune response like what occurs with myeloperoxidase (35,36). Then, the increased CAT activity observed in plasma during the recovery of scuba diving could have a neutrophilic origin.

It has been evidenced that a single HBO induces oxidative DNA damage (22). In this sense, the role of extracellular SOD, as protector against oxidative stress, could be related to an attenuation of renal p22 (phox) expression, NADPH oxidase activation, and the accompanying renal vasoconstriction and hypertension (46). However, it can also take into account that EC-SOD spares NO, and then, the NO function is enhanced. However, the physiological relevance of elevations in NO concentration due to hyperoxia requires more investigation. These changes may contribute to angiogenesis augmentation and inhibition of neutrophil β_2-integrin function that have been reported with hyperbaric O_2 (34,43).

In conclusion, plasma and erythrocytes show a response to scuba diving session at a 40-m depth increasing the ROS detoxifying enzymes to avoid the appearance of oxidative damage. This antioxidant response also spares NO to facilitate its vasodilatory function and to decrease the number of venous gas bubbles in the postdive period.

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Conflict of Interest: None

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES

AQ1 = Please provide the middle initials of the following authors: Antoni Sureda, Pedro Tauler, and Antoni Pons.

AQ2 = Please provide location of Bayer.

AQ3 = Please provide the name of the manufacturer of Shimadzu UV-2100 spectrophotometer and its location.

AQ4 = Please provide location of Cell Biolabs.

AQ5 = Please spell out HRP.

AQ6 = Please provide the name of the manufacturer of SPSS 14.0 for Windows and its location.

AQ7 = Please check if changing Fl to fL [femtoliters] is correct.

AQ8 = Please check if changing Pg to pg [picogram] is correct.

AQ9 = GPer versus Gper; the former was followed. Please check if this is correct.

AQ10 = Please check if changes in the following statement reflect the intended meaning: "It was evidenced that when antioxidant power was increased (after 4 wk of antioxidant supplementation), the negative effects of a scuba diving on endothelial function were attenuated."

AQ11 = Please spell out EC; this was not mentioned anywhere in the text.

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