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Neutrophil Tolerance to Oxidative Stress Induced by Hypoxia/Reoxygenation

ANTONI SUREDA[a], JOAN M. BATLE[b], PERE TAULER[a], NURIA CASES[a], ANTONI AGUILÓ[a], JOSEP A. TUR[a] and ANTONI PONS[a,*]

[a]*Laboratori de Ciències de l'Activitat Física, Departament de Biologia Fonamental i Ciències de la Salut, Universitat de les Illes Balears, Campus Universitari, Crtra. Valldemossa, Km 7.5, E-07122-Palma de Mallorca, Illes Balears, Spain;* [b]*MEDISUB, C/Alcanada, Alcúdia, Illes Balears, Spain*

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Repetitive episodes of hypoxia/reoxygenation induce cellular adaptations resulting in a tolerance process against oxidative stress. We studied the effects of chronic episodes of hypoxia/reoxygenation on neutrophil antioxidant defenses, neutrophil oxidative capability, and oxidative damage induced in neutrophils and plasma. Seven professional apnea divers participated in the study. Blood samples were taken under basal conditions, after a diving apnea session, and under basal conditions after five consecutive days of diving apnea sessions (basal post-diving). Chronic episodes of hypoxia/reoxygenation increased malondialdehyde (MDA), carbonyl derivates and creatine kinase (CPK) in plasma. Neutrophil catalase (CAT) levels were higher in basal post-diving. Neutrophil oxidative burst was maintained after diving, although the maximum response was delayed in basal post-diving. Neutrophil thioredoxin reductase (TR) activity increased in basal post-diving, and glutathione reductase (GR) activity was maintained. Chronic, repetitive episodes of diving apnea induce neutrophil adaptations in order to delay the oxidative burst response and to facilitate protein reduction. Diving apnea could be a good model to study tolerance to the oxidative stress generated by hypoxia/reoxygenation.

Keywords: Oxidative stress; Oxidative tolerance; Antioxidants; Neutrophil; Hypoxia/Reoxygenation; Diving apnea

Abbreviations: ALT, alanine transaminase; ANOVA, analysis of variance; APIR, acute phase immune response; AST, aspartate transaminase; CAT, catalase; CL, luminol chemiluminescence; CPK, creatine kinase; DNPH, 2,4-dinitrophenylhydrazine; GGT, glutamil transpeptidase; GR, glutathione reductase; LDH, lactate dehydrogenase; MPO, myeloperoxidase; NO, nitric oxide; OZ, opsonized zymosan; ROS, reactive oxygen species; SOD, superoxide dismutase; TR, thioredoxin reductase

INTRODUCTION

Circulating neutrophils are important in tissue surveillance, repair and adaptation of human skeletal muscle. Interactions between neutrophils and muscle may play a significant role in modulating the course of muscle injury and repair after oxidative damage.[1] Although activated neutrophils present increased capabilities to synthesize reactive oxygen species (ROS) that could produce oxidative damage,[2,3] this increase in oxidative activity could inhibit neutrophil functions by auto-oxidative processes.[4,5] Long-duration or damaging exercise increases oxygen consumption and causes a disturbance between intracellular pro-oxidant and antioxidant homeostasis.[2,3] Several studies have investigated the ROS producing activity of circulating neutrophils after exercise by luminol-dependent chemiluminescence (CL) with controversial results.[6–8] Luminol CL mainly detects the MPO-dependent formation of HOCl. In most cases, the luminol-dependent CL response was augmented after exercise,[8] but repeated exercise sessions could reduce the CL.[9] Exhaustive exercise could inhibit neutrophil response to exogenous stimuli. This impaired neutrophil function may lead to a predisposition to infection after extensive exercise.[10] The role of neutrophils after exercise is not exactly known.

*Corresponding author. Tel.: +34-971173171. Fax: +34-971173184. E-mail: dbsapb0@uib.es

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Hypoxia/reoxygenation induces significant changes in the endogenous antioxidant system and increases the production of ROS.[11,12] Cellular hypoxia and reoxygenation participate in injury caused by ischemia/reperfusion and in human diseases such as sleep apnea, circulatory shock, myocardial ischemia, stroke, and organ transplantation.[13,14] Diving apnea subjects the organism to successive, intermittent episodes of hypoxia and reoxygenation, increasing the levels of circulating neutrophils and decreasing the GSH/GSSG ratio, as also that occurs after exhaustive exercise. The neutrophil response to diving apnea resembles the acute phase immune response (APIR) to infection.[4,5] It has been suggested that this inflammatory-like immune response is necessary for muscular regeneration and adaptation to physical exercise.[15,16] Diving apnea could be a good model to study the role of neutrophils in the inflammatory response related to the cellular injury associated to several human diseases.

It has been demonstrated that induction of oxidative stress with ischemia/reperfusion renders the cells more resistant to the detrimental effects induced by a second ischemia/reperfusion episode. Exposing endothelial cells to repetitive hypoxia/reoxygenation episodes results in a tolerance process with respect to neutrophil adhesion to the endothelium by increasing NO production.[17] This tolerance reduces the oxidative stress induced by hypoxia/reoxygenation, and requires hours or days to develop.[17] Cellular antioxidant systems have demonstrated a great adaptation to chronic exercise[18] to avoid the oxidative damage caused by ROS produced during exercise. The neutrophil tolerance mechanisms to oxidative stress are as yet unknown. Diving apnea could be an useful model to study the neutrophil adaptation and tolerance to a chronic hypoxia/reoxygenation situation in a similar way as it occurs in sleep apnea.

The aim of this work was to study the effects of chronic episodes of hypoxia/reoxygenation, such as repetitive diving apnea sessions over consecutive days, on both neutrophil antioxidant defenses, on the neutrophil capacity to produce ROS and on the oxidative damage produced in neutrophils and plasma.

MATERIALS AND METHODS

Subjects

Seven voluntary male subjects participated in this study. They were all professional apnea divers. The study took place in the Spanish Championship of apnea fishing 2002 in Pollença (Balearic Islands). Subjects were informed of the purpose of this study and the possible risks involved before giving their written consent to participate. The participants performed three days of hard training and two days of competition. The characteristics of a training-day and the competition-days are indicated in Table I. We monitored the last training-day and the two days of competition using a dive computer.

TABLE I Characteristics of the diving apnea sessions

	Training day	Competition day 1	Competition day 2
Total apnea session time (min)	194 ± 24	250 ± 11	118 ± 6
Number of immersions	58.4 ± 8.8	105 ± 24	43.0 ± 11.0
Mean depth (m)	19.6 ± 0.7	25.2 ± 2.2	26.0 ± 4.6
Total immersion time (min)	75.4 ± 4.3	121 ± 7	44.6 ± 6.5
Total surface time (min)	119 ± 21	128 ± 9	73.0 ± 9.2

Characteristics of one training day and the two competition days. Means ± SEM.

Experimental Procedure

Venous blood samples were taken from the antecubital vein with suitable vacutainers with EDTA as anticoagulant. Samples were taken in basal conditions, the morning previous to the beginning of the training sessions (pre-diving basal), 4 h after ending the competition (4 h post-diving) and the following morning in basal conditions (post-diving basal). Blood samples were used to purify neutrophils and to obtain plasma. The enzyme activities of glutathione reductase (GR), thioredoxin reductase (TR), myeloperoxidase (MPO), and the protein levels of catalase (CAT), and Cu, Zn superoxide dismutase (Cu,ZnSOD) were determined in neutrophils. Protein carbonyl derivates were measured in plasma and neutrophils, and malondialdehyde (MDA) was measured in plasma. Creatine kinase (CPK), aspartate transaminase (AST), alanine transaminase (ALT), glutamil transpeptidase (GGT) and lactate dehydrogenase (LDH) were measured in serum. The oxidative capacity of neutrophils was determined by luminol CL.

Neutrophil Purification

Neutrophil fraction was purified following an adaptation of the method described by Boyum.[19] Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and then, it was centrifuged at 900*g*, at 4°C for 30 min. The precipitate containing the 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 (1:10) for enzymatic and carbonyl analysis or resuspended in Hank's balanced salt solution (HBSS) for CL assays.

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

Enzymatic Determinations

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

GR activity was measured by a modification of the Goldberg and Spooner[20] method. This assay required oxidised glutathione as the substrate.

TR activity was measured with an end-point method by thioredoxin coupled insulin reduction assay.[21] Absorbance at 412 nm was determined after incubation at 37°C for 20 min.

MPO activity of neutrophils was measured by guaiacol oxidation.[22] 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.

ELISA Determination of CAT and SOD

CAT and SOD levels were determined by ELISA using polyclonal antibody Anti CAT (Calbiochem) which recognizes human CAT and polyclonal antibody Anti Cu,ZnSOD (Sigma) which recognizes human Cu,ZnSOD. We followed an adaptation of the method previously described.[23]

Suitable dilutions of the neutrophil suspensions or standard dilutions of CAT and SOD (50 μl) were placed in each well of the plate per duplicate (Polystyrene Assay Plate, Costar). The plate was then incubated (37°C for 3 h) in order to adsorb the sample proteins to the surface of each well. 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. After that the plate was washed four times with NaCl 0.9%–Tween 20. The primary antibody (1:1000) was placed in 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 (1:500) was placed in each well and the plate was incubated in the same condition. The wells were newly washed and the phosphatase substrate solution was added. Finally, absorbance was measured at 405 nm.

Protein Carbonyl Derivate Determination in Neutrophils

Carbonyl derivates were measured by an adaptation of the method of Levine.[24] Samples were deproteinised with trichloroacetic acid. Precipitates were resuspended with 2,4-dinitrophenylhydrazine (DNPH) 10 mM, and incubated for 60 min at 37°C. Then, samples were precipitated with 20% trichloroacetic acid, and centrifuged for 10 min at 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 carbonyl. Samples were analysed against a blank of guanidine solution.

MDA Levels in Plasma

Plasma MDA as a marker of lipid peroxidation was analyzed by a colorimetric assay kit (Calbiochem, San Diego, CA, USA).

CL 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). CL was measured at 37°C for 90 min in FL × 800 Microplate Flurescence Reader (Bio-tek Instruments, Inc.). Each sample was determined in duplicate.

Serum Enzyme Activities

Determinations were made using commercial clinical kits in an autoanalyser system (Technicon DAX System). In the determination of CPK activity the enzyme reacts with creatine phosphate and ADP to form ATP, which is coupled to the hexokinase/GDP reaction generating NADPH.[25] The AST produces oxalacetate in the reaction between L-aspartate and 2-oxoglutarate, and the oxalacetate formed is reduced to malate with concomitant oxidation of NADH catalyzed by malate dehydrogenase.[26] ALT catalyzes the reaction between L-alanine and

2-oxoglutarate, and the pyruvate formed is reduced by NADH in a reaction catalyzed by LDH.[27] The LDH activity determination is based on the measurement of the conversion of pyruvate to L-lactate by monitoring the oxidation of NADH.[28] All these activities were monitored by measuring the change in absorbance at 340 nm. GGT reacts with the synthetic substrate (L-γ-glutamyl-3-carboxy-4-nitroanilide) and glycylglycine acts as an acceptor for the γ-glutamyl residue and 5-amino-benzoate is liberated.[29] The rate of formation was measured at 410 nm.

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. One-way ANOVA was used to determine the significance of changes, in all parameters measured, induced by the repetitive diving apnea sessions.

RESULTS

The characteristics of the three diving apnea training sessions were similar. Table I shows the characteristics of the last training-day and the two days of competition. Training sessions were performed at lower depth than competition. During the first day of competition, there were a greater number of immersions and the apneas lasted longer (2 h) compared to the last day of competition (45 immersions and 45 min in apnea) and the three training-days (60 immersions and 75 min in apnea). Overall the competition plus training submitted the divers to about 6.5 h of apnea and 327 immersions.

Repetitive diving apnea sessions maintained the basal pre-diving circulating leukocytes; however, there was an increase in the number of circulating neutrophils 4 h after the end of the last diving session. Then, the circulating neutrophils returned to the basal value (Table II). Plasma MDA and plasma protein–carbonyl derivates, as markers of oxidative damage, maintained the pre-diving basal value after the last diving apnea session, and increased 3.7 and 1.5 times, respectively, in plasma basal post-diving compared to the plasma pre-diving basal value; however, neutrophils protein–carbonyl derivates always maintained the pre-diving basal value. AST, ALT, GGT, LDH serum activities, as markers of tissue damage, were maintained at the pre-diving basal level; however, CPK activity significantly increased about 2.4 times in serum obtained 4 h after the last diving session and it dropped 1.9 times in the post-diving basal serum (Table II).

TABLE II Effects of diving apnea on leukocyte, neutrophil levels and oxidative damage

	Pre-diving basal	4 h Post-diving	Post-diving basal
Leukocytes (10^3 cells/μl)	6.08 ± 0.97	8.69 ± 1.05	5.56 ± 0.61
Neutrophils (10^3 cells/μl)	3.71 ± 0.79	5.95 ± 0.86*	3.03 ± 0.39▽
Plasma MDA (μmol/l)	5.44 ± 0.50	9.22 ± 1.75	20.3 ± 3.4*▽
Plasma carbonyl derivates (μmol/l)	327 ± 15	343 ± 16	481 ± 21*▽
Neutrophil carbonyl derivates (μmol/10^9 cells)	8.21 ± 1.32	6.75 ± 0.56	8.66 ± 0.75
CPK (U/l)	126 ± 10	301 ± 58*	236 ± 45*
AST (U/l)	16.6 ± 0.7	20.0 ± 1.3	19.4 ± 2.0
ALT (U/l)	18.1 ± 1.2	22.3 ± 1.8	22.8 ± 1.6
GGT (U/l)	13.4 ± 0.7	16.0 ± 2.6	13.7 ± 1.5
LDH (U/l)	322 ± 9	340 ± 15	341 ± 22

One-way ANOVA. (*) Indicates significant differences between groups versus initial basal value, (▽) or versus 4 h post-diving value. Means ± SEM.

CAT and SOD maintained the pre-diving basal protein levels in neutrophils obtained 4 h after the last diving session (Table III). The neutrophil post-diving basal CAT levels were about 2.4 times significantly higher than the pre-diving basal, while neutrophil SOD levels remained at the pre-diving basal level. The neutrophil MPO pattern was different to the neutrophil CAT pattern. The neutrophil MPO pre-diving basal activity was maintained 4 h after the last apnea diving session but significantly dropped to 40% of activity in post-diving basal. The CL of neutrophils produced after their activation with zymosan was always maintained at the pre-diving basal value. However, there were differences in the time at which the maximal CL level was attained: the post-diving neutrophils

TABLE III Effects of diving apnea on neutrophil antioxidant enzyme levels and oxidative capacity

	Pre-diving basal	4 h Post-diving	Post-diving basal
SOD (ng/10^3 cells)	11.1 ± 2.4	8.26 ± 0.9	13.8 ± 1.3
Catalase (ng/10^3 cells)	0.378 ± 0.09	0.270 ± 0.04	0.915 ± 0.08*▽
MPO Activity (nKat/10^9 cells)	173 ± 18	215 ± 59	71.9 ± 7.2*▽
Luminol chemiluminescence			
RLU max/ 10^6 cells	3389 ± 364	3175 ± 319	3236 ± 241
Time of max RLU (s)	1338 ± 21	1544 ± 55	1661 ± 102*

One-way ANOVA. (*) Indicates significant differences between groups versus initial basal value, (▽) or versus 4 h post-diving value. Means ± SEM.

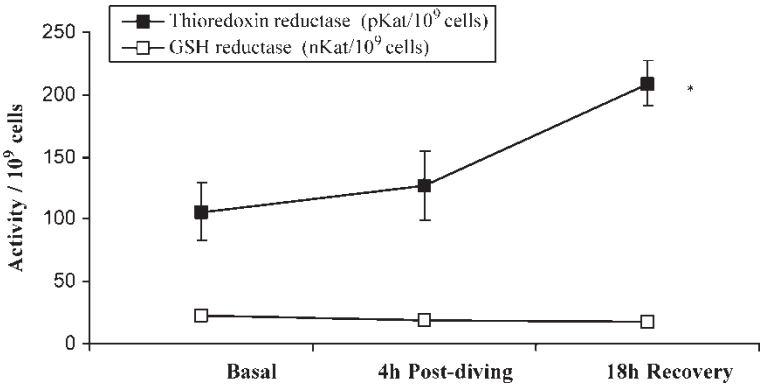

FIGURE 1 Effects of diving apnea on neutrophil enzyme activities. Thioredoxin reductase (■) expressed as pKat/10^9 cells and glutathione reductase (□) expressed as nKat/10^9 cells. One-way ANOVA. (*) Indicates significant differences between groups versus initial basal value, (▽) or versus 4 h post-diving value. Means ± SEM.

significantly delayed the maximum CL response to zymosan about 330 s versus the pre-diving neutrophils.

Figure 1 shows the effects of diving apnea sessions on thioredoxin and GR activities in neutrophils. The neutrophil pre-diving basal levels of GR were maintained in all situations analyzed; however, post-diving basal TR activity in neutrophils was about 2 times higher than the pre-diving basal value as well as the 4 h post-diving value.

DISCUSSION

The diving apnea sessions are similar to those presented in other studies.[30] These diving apnea sessions produce an APIR after diving and install an oxidative stress in neutrophils.[30,31] ROS production is increased in the reduced state that characterizes cellular hypoxia, and oxidants are produced in excess during reoxygenation.[32,33] O_2 deprivation during diving apnea could increase muscle damage. Increased levels of CPK in serum after diving and the maintenance of these high values in the basal post-diving could be an indicator of muscle repair.[34] CPK together with other released substances by the injured muscle could act initiating inflammation.[35] Invading neutrophils could phagocyte cellular debris and release growth factors that recruit other inflammatory cells such as macrophages involved in removing residual cell fragments and in reconstructing the muscle fiber.[15,36,37]

The neutrophilia present after diving apnea suggests the presence of an APIR similar to the one induced by infection, by intense exercise[4,23] or by ischemia/reperfusion.[38] During the APIR, neutrophils are primed to the oxidative burst.[8,39] The activated neutrophils obtained after diving and post-diving basal produce similar CL intensity than initial basal. However, neutrophil oxidative burst in response to exogenous stimuli, such as OZ, is delayed in the post-diving basal situation, parallel to a lower neutrophil MPO activity observed in activated neutrophils. Luminol mainly detects hypochlorous acid generated by MPO activity.[40] Neutrophil azurophilic granules contain large quantities of MPO, which are discharged in response to exogenous stimuli and reacts with H_2O_2 and Cl^- producing hypochlorous acid. Neutrophil post-diving basal MPO activity is lower, and could be related to the slower response of neutrophils to the OZ stimuli. Chronic, repetitive episodes of hypoxia/reoxygenation could adapt the oxidative machinery of neutrophils in order to decrease the sensitivity against the stimuli, avoiding autoimmune responses.

The increase in the TR activity and CAT levels in post-diving basal could evidence the oxidative tolerance of neutrophils to oxidative stress induced by hypoxia/reoxygenation. TR plays an important role in the defense against oxidative stress by reducing disulfide sites in oxidized proteins. The higher TR post-diving basal activity could indicate a neutrophil response to counteract the oxidation that takes place as a consequence of diving apnea. The importance of this adaptation could be evidenced by the decrease in the viability of murine endothelial cells cultured in thiol-free medium after hypoxia/reoxygenation, but injury was diminished by exogenous thioredoxin.[41] GR is also important because it recycles glutathione in order to maintain the cellular redox status; however, this activity is maintained at the pre-diving level in all situations. CAT—the main enzyme to eliminate high H_2O_2 concentration[42]—presents higher post-diving basal levels than in pre-diving. Hence, both TR activity and CAT levels could protect neutrophils from the oxidative stress generated by hypoxia and could potentially reduce the deleterious effects of the oxidative burst. The effectiveness of this adaptation could be evidenced by the maintenance of the carbonyl index values in neutrophils after diving. Neutrophil antioxidant defenses are able to avoid

oxidative damage in neutrophils caused by repetitive, chronic diving apnea episodes. However, the post-diving basal plasma MDA and protein carbonyl levels are higher. Different studies have observed that plasma lipid peroxidation induced by acute exercise increases more during hypoxia than in normoxia.[43] Neutrophil adaptation to hypoxia/reoxygenation does not avoid plasma protein and lipid damage.

The oxidative tolerance induced in neutrophils in the post-diving situation could contribute to repair the muscle damage induced by hypoxia/reoxygenation in diving apnea. However, oxidative adaptation is unable to avoid the appearance of oxidative products in plasma.

In summary, chronic episodes of diving apnea produce an adaptation or oxidant tolerance in neutrophils that could protect neutrophils from the oxidative stress generated by hypoxia/reoxygenation, and could facilitate the neutrophil contribution to muscle repair.

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