The Physiology and Pathophysiology of the Hyperbaric and Diving Environments

DNA damage after long-term repetitive hyperbaric oxygen exposure

Michael Gröger,1 Süükrü Öter,1,4 Vladislava Simkova,1,5 Markus Bolten,2 Andreas Koch,2 Volker Warninghoff,2 Michael Georgieff,1 Claus-Martin Muth,1 Günter Speit,3 and Peter Radermacher1

1Sektion Anaesthesiologische Pathophysiologie und Verfahrensentwicklung and 2Abteilung Humangenetik, Universitätsklinikum, Ulm; 3Schifffahrtsmedizinisches Institut der Marine, Kronshagen, Germany; 4Fizyoloji Anabilim Dali, Gülhane Askeri Tip Akademisi, Ankara, Turkey; and 5Anesteziologicko-resuscitacni klinika, Fakultni nemocnice u sv Anny, Brno, Czech Republic

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Gröger M, Öter S, Simkova V, Bolten M, Koch A, Warninghoff V, Georgieff M, Muth CM, Speit G, Radermacher P. DNA damage after long-term repetitive hyperbaric oxygen exposure. J Appl Physiol 106: 311–315, 2009. First published November 20, 2008; doi:10.1152/japplphysiol.90737.2008.—A single exposure to hyperbaric oxygen (HBO), i.e., pure oxygen breathing at supra-atmospheric pressures, causes oxidative DNA damage in humans in vivo as well as in isolated lymphocytes of human volunteers. These DNA lesions, however, are rapidly repaired, and an adaptive protection is triggered against further oxidative stress caused by HBO exposure. Therefore, we tested the hypothesis that long-term repetitive exposure to HBO would modify the degree of DNA damage. Combat swimmers and underwater demolition team divers were investigated because their diving practice comprises repetitive long-term exposure to HBO over years. Nondiving volunteers with and without endurance training served as controls. In addition to the measurement of DNA damage in peripheral blood (comet assay), blood antioxidant enzyme activities, and the ratio of oxidized and reduced glutathione content, we assessed the DNA damage and superoxide anion radical (O2•−) production induced by a single ex vivo HBO exposure of isolated lymphocytes. All parameters of oxidative stress and antioxidative capacity in vivo were comparable in the four different groups. Exposure to HBO increased both the level of DNA damage and O2•− production in lymphocytes, and this response was significantly more pronounced in the cells obtained from the combat swimmers than in all the other groups. However, in all groups, DNA damage was completely removed within 1 h. We conclude that, at least in healthy volunteers with endurance training, long-term repetitive exposure to HBO does not modify the basal blood antioxidant enzyme capacity or the basal level of DNA strand breaks. The increased ex vivo HBO-related DNA damage in isolated lymphocytes from these subjects, however, may reflect enhanced susceptibility to oxidative DNA damage.

combat swimmers; underwater demolition team divers; endurance training; comet assay; superoxide radical; superoxide dismutase; catalase; glutathione peroxidase

EXPOSURE TO HYPERBARIC OXYGEN (HBO), i.e., pure oxygen breathing at supra-atmospheric pressures, increases the formation of oxygen radical species (21, 27), which in turn results in consumption of antioxidants (2, 23) and reduces antioxidant enzyme activity (4), ultimately causing lipid peroxidation (3, 4, 26, 31), organ injury (5), and DNA damage (10, 11, 17, 20, 26, 32–37, 39–42). On the other hand, HBO was reported to promote protective preconditioning against ischemia-reperfusion-induced oxidative organ injury in the brain, spinal cord, heart, and liver (16, 28, 45, 46). Furthermore, in healthy volunteers, the HBO-induced DNA damage not only rapidly disappeared after the end of the HBO exposure, but a subsequent exposure did not cause oxidative DNA damage any more (10), indicating the induction of antioxidant defense (36, 40, 41) that lasted for at least 1 wk (32). In fact, both a single HBO exposure as well as a compressed-air dive to a depth of 40 m, i.e., at an inspiratory O2 partial pressure (PO2) of 1 bar, increased the lymphocyte glutathione peroxidase activity (15). Finally, in healthy volunteers with a long-term diving experience and, consequently, repetitive diving-associated exposures to hyperoxia, HBO-induced DNA damage was less pronounced than in nondiving controls (11, 26).

Therefore, we tested the hypothesis that long-term repetitive exposure to HBO would modify the degree of DNA damage induced by a single ex vivo HBO exposure of isolated human peripheral lymphocytes. In addition, we investigated whether long-term repetitive exposure to high inspiratory PO2 values would affect the lymphocyte total cellular superoxide radical (O2•−) production in response to a single, well-defined HBO-induced oxidative stress. We studied combat swimmers and underwater demolition team (UDT) divers because, over several years, these subjects perform dives breathing pure O2 and O2-enriched inspiratory gas mixtures using closed and semi-closed circuits, respectively. Thus they represent a population with a particularly long-term repetitive exposure to HBO. Combat swimmers and UDT divers undergo pronounced endurance training, and most of their diving activity also comprises strenuous exercise. Exhaustive physical effort per se induces oxidative stress-related DNA damage (14, 18, 19, 25, 29), particularly beyond the anaerobic threshold (14, 18). Since this response is markedly affected by the degree of endurance training (29), a group of “Naval Pentathlon” athletes was also studied to compare the divers with nondiving subjects with a comparable degree of endurance training. Finally, isolated peripheral lymphocytes were investigated because J) isolated lymphocytes show more pronounced HBO-induced DNA damage than whole blood samples and represent a well-established model of human lymphoid tissue.

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model for the investigation of DNA damage in human bio-
monitoring (43), and 2) they allow for the use of HBO at P O2 values and duration beyond those of the so-called “oxygen tolerance test” (23), i.e., HBO at 2.8 bar for 30 min. The in vivo central nervous O2 toxicity (6) otherwise precludes exposing human volunteers to these HBO exposures. The level of HBO-induced DNA damage was assessed using the comet assay (single-cell gel electrophoresis), which allows measure-
ment of the induction of DNA damage as well as its repair (43). Previous investigations using the comet assay clearly demon-
strated induction of oxidative DNA damage (including oxida-
tive DNA base damage as detected by the FPG protein) in lymphocytes after exposure to a single HBO in vivo and in vitro as well as rapid repair of the induced lesions (39).

METHODS

The present study was part of a larger investigation entitled “Ox-
idative Stress during Hypoxygenation and Diving,” the protocol of which had been approved by the ethical committee of the Christian-Al-
brecht-Universität (Kiel, Germany). All subjects gave their written informed consent to participate in the investigation. A total of 37 subjects were studied comprising combat swimmers (n = 6), UDT divers (n = 13), and nondiving volunteers either with (Naval Pen-
tathlon athletes, n = 6) and without endurance training (controls, n = 12). The demographic data of the subjects are summarized in Table 1. The UDT divers had a diving experience of several years with a diving activity of ~10 h using O2-enriched gas mixtures and closed-
circuit pure O2 breathing apparatus during the 15 mo preceding the study (Table 1). The combat swimmers had a median diving experience using a closed-circuit pure O2 breathing apparatus of nearly 1 yr with a median diving time using this device of ~100 h (Table 1). The UDT divers and combat swimmers had performed their last dives using O2-enriched gas mixtures or closed-circuit pure O2 breathing apparatus 4–6 and 2 wk, respectively, before the investigation began. The Naval Pentathlon athletes were investigated during their world championship preparation, which had started 2 mo before the investi-
gation. All investigations took place during a 2-wk period. Blood sampling took place in the morning after overnight fasting and was part of the individual subjects’ regular routine medical examination.

Whole blood measurements. In all subjects, whole blood samples were analyzed for superoxide dismutase (SOD), catalase, and plasma glutathione peroxidase (GPx) activities as well as the concentrations of reduced (GSH) and oxidized (GSSG) glutathione, respectively, using commercially available test kits as described previously (3) (SOD: SOD assay kit-WST, Dojindo Molecular Technologies, Kumamoto, Japan; catalase: Bioxytech Catalase-520, OxirResearch, Portland, OR; GPx: glutathione peroxidase assay kit, Cayman Chemical, Ann Arbor, MI; GSH and GSSG: GSH/GSSG ratio assay kit, Calbio-
chem, EMD Chemicals, San Diego, CA). DNA strand breaks were quantified with the alkaline version of the comet assay (44). Briefly, cell lysis for at least 1 h and slide processing were performed as previously described in detail (17, 20, 26) using alkali denaturation and electrophoresis (0.86 V/cm at a pH > 13) to transform alkali-
sensitive parts of the DNA into DNA strand breaks. Measurements were made by image analysis, determining the mean tail moment and the mean tail intensity of 100 cells per slide (2 slides each per measurement in each individual). In none of the experiments was there a relevant difference between these two parameters. Therefore, in continuation of our previous studies, we chose the tail moment for the presentation of our results.

Isolated lymphocytes. Lymphocytes were separated on Ficoll gra-
dients, washed in PBS buffer, resuspended in RPMI 1640 buffer, and thereafter exposed to HBO in a hyperbaric chamber containing 2% CO2 (to maintain pH 7.0–7.5) and 98% O2 as described previously (37°C, compression and decompression rate 0.2 bar/min, 2-h isopres-
sion at 4 bar) (20). The comet assay was performed in aliquots before as well as immediately, 1, and 2 h after the HBO exposure. In addition, the lymphocyte total cellular O2•− concentration was measured at room temperature after incubation with the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) before and immediately after the HBO exposure by electron spin resonance using a Bruker EMX spectrometer (12). Results are expressed as nanomoles of O2•− released by 106 cells. To exclude any effect of the lymphocyte incubation procedure on the comet assay results and the electron spin resonance measurements per se, we also determined the tail moment and the O2•− in time-control lymphocyte aliquots without HBO exposure (n = 6).

Statistics. All data are presented as median (quartiles, with range in parentheses) unless otherwise stated. After the exclusion of normal distribution of the main criteria (tail moment) using the Kolmogorov-
Smirnov test, differences between the four groups were tested using a Kruskal-Wallis one-way analysis of variance on ranks and a subsequent Dunn test. The time course of the tail moment in the isolated lymphocytes before and after HBO exposure was tested using a Friedman repeated-measures analysis of variance on ranks and a subsequent Dunn test, and the difference between the lymphocyte O2•− release before and after the HBO exposure was tested using a Wilcoxon signed rank test.

RESULTS

Table 2 summarizes the results of the whole blood DNA damage (tail moment), antioxidant enzyme activity, and glutathione measurements. There were no statistically significant intergroup differences in any of these parameters.

Figures 1 and 2 summarize the results of the ex vivo HBO exposure of the isolated lymphocytes. The tail moment of time-control lymphocyte preparations (n = 6) without HBO exposure [0.10 (0.09, 0.11), 0.12 (0.08, 0.12), 0.11 (0.09, 0.12), and 0.10 (0.08, 0.12) at 0, 2, 3, and 5 h, respectively, which correspond to the time points before and immediately, 1, and

Table 1. Demographic data of the subjects

<table>
<thead>
<tr>
<th>No. of Subjects</th>
<th>Age, yr</th>
<th>Weight, kg</th>
<th>Height, cm</th>
<th>VO2 max, ml·min−1·kg−1</th>
<th>Diving Experience With Pure O2 or O2-Enriched Gas Mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>12</td>
<td>25 (18, 47)</td>
<td>78 (58, 109)</td>
<td>181 (165, 191)</td>
<td>ND</td>
</tr>
<tr>
<td>Naval Pentathlon athletes</td>
<td>6</td>
<td>29 (22, 37)</td>
<td>76 (70, 82)</td>
<td>178 (173, 192)</td>
<td>74 (73, 77)*</td>
</tr>
<tr>
<td>UDT divers</td>
<td>13</td>
<td>29 (22, 48)</td>
<td>90 (66, 105)</td>
<td>185 (163, 194)</td>
<td>45 (28, 59)</td>
</tr>
<tr>
<td>Combat swimmers</td>
<td>6</td>
<td>25 (24, 28)</td>
<td>80 (64, 92)</td>
<td>178 (175, 190)</td>
<td>48 (42, 60)</td>
</tr>
</tbody>
</table>

Demographic data and maximum O2 uptake (VO2 max) are median values (with range in parentheses) in the nondiving subjects without (controls) and with endurance training (Naval Pentathlon athletes), underwater demolition team (UDT) divers, and combat swimmers. * P < 0.05 vs. other groups; ND, not determined. Note that there was no intergroup difference for age, sex, height, and weight except for the markedly higher anaerobic threshold (VO2 max) in the Naval Pentathlon athletes.
HBO comparably increased SOD, superoxide dismutase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione.

The key findings were that there was no intergroup difference in the incidence of blood cell DNA damage or in antioxidant enzyme activity or capacity, 2) ex vivo HBO exposure was associated with more pronounced O$_{2}^{•−}$ formation in isolated lymphocytes from the UDT divers and combat swimmers than in those from control subjects and Naval Pentathlon athletes, and 3) lymphocytes from combat swimmers showed the most pronounced increase in HBO-induced DNA damage, which in turn was rapidly removed within 1 h.

Air breathing under hyperbaric conditions results in exposure to hyperoxia, since according to Boyle’s law, the inspira-
tory PO2 increases in direct proportion to the rise in ambient pressure. Nevertheless, although ample literature is available demonstrating that both hyperbaric and prolonged normobaric pure O2 breathing cause oxidative DNA damage (10, 11, 17, 32–37, 39–42), lipid peroxidation, and reduction of both enzymatic and nonenzymatic antioxidant capacity (1, 2, 4, 23, 27, 31), contradictory results have been reported on the effect of hyperoxia associated with diving per se: after a dry chamber dive to a simulated depth of 250 m with maximum inspiratory PO2 of 70 kPa, Djurhuus et al. (13) found a decrease in the blood content of reduced GSH, whereas no DNA single-strand breaks could be detected. In contrast, both a water immersion to 40-m breathing air (i.e., at an inspiratory PO2 of 100 kPa) and a dry chamber HBO exposure to 2.2 bar (i.e., at an inspiratory PO2 of 220 kPa) not only caused increased lymphocyte H2O2 production but also resulted in enhanced intracellular GPx activity and, in particular, expression of heme oxygenase-1 (HO-1) mRNA (15). The latter finding is of particular interest: both in vivo and in vitro, enhanced HO-1 expression assumes crucial importance as an adaptive protection against HBO-induced oxidative DNA damage (17, 35–37).

Our finding that before any HBO exposure, blood samples from the four different groups studied presented with a comparable degree of DNA damage and red blood cell or plasma antioxidant capacity confirms data described by other authors as well as our own group: Niess et al. (29) demonstrated that the tail moment in whole blood samples obtained from well-trained long-distance runners before an exhaustive treadmill exercise was similar to that in sedentary control subjects. We previously reported that before an exposure to HBO, experienced divers (26) showed a level of DNA damage in whole blood samples nearly identical to that in nondiving volunteers (10, 11). Complementary to these observations, other authors reported that SOD, catalase, and GPx activities as well as blood GSH content and the GSH/GSSG ratio did not differ between trained subjects and untrained controls (30, 38). Furthermore, divers with long-term diving activity also presented with values for these parameters similar to those for nondivers (10, 11, 26). Nevertheless, our present observations contrast with data reported by Knez et al. (22), who found significantly higher resting catalase and GPx activities and lower malondialdehyde concentrations in ironman triathletes than in matched control subjects. We can only speculate on this discrepancy, particularly since the training level of the Naval Pentathlon athletes in our study [maximum VO2 = 74 (range 73–77) ml·min⁻¹·kg⁻¹] was even slightly higher than that reported to induce an adaptive increase in antioxidant enzyme activity in other endurance athletes, i.e., ironman triathletes, by other authors [maximum VO2 = 65 ± 6 (22) and 66 ± 4 ml·min⁻¹·kg⁻¹ (30), respectively].

Prolonged normobaric hyperoxia was shown to cause oxidative stress, which is associated with DNA damage (7, 8) that can be detected using the comet assay (44). Short-term exposure to HBO leads to comparable oxidative stress, and thus it is used as an appropriate model to investigate oxidatively generated DNA damage and its repair (41). In good agreement with this rationale, exposure of isolated lymphocytes to HBO induced DNA damage in our experiment, and this response was twice as high in the blood samples obtained from the combat swimmers than in all the other groups. Hence, our data suggest that HBO exposure induced more DNA damage in these subjects than in the UDT divers, nondiving endurance athletes, and nondiving sedentary controls. On the other hand, background levels of DNA damage were measured in these samples 1 h after HBO exposure. Near-complete removal of the HBO-induced DNA damage within 1 h was also observed in the samples from the combat swimmers despite the higher levels of DNA damage immediately after the HBO exposure. It is unclear whether this finding indicates higher DNA repair capacity of lymphocytes from combat swimmers, because no measurements were performed at earlier time points. It should be noted that due to the duration of the HBO exposure itself (2 h of isopression plus 20 min each of compression and decompression, respectively), it is impossible to determine any influence of DNA repair on the comet assay effects during this period of time and to establish exact repair kinetics. Our results are in agreement with previous findings that also reported rapid repair of DNA damage in human lymphocytes after exposure to HBO in vivo and in vitro (39).

Interestingly, there was no difference in the removal of DNA damage after the end of the HBO exposure between the Naval Pentathlon athletes and the sedentary control subjects. Since the HBO-related O2•− production did not differ between these two groups, these findings suggest that, at least under our experimental conditions, endurance-trained athletes and sedentary controls have a nearly identical response to HBO-induced oxidative stress. This result is in contrast to literature reports on the effect of exhaustive effort in trained and untrained subjects: depending on the type of activity, both increased and decreased antioxidant enzyme activities and increased levels of DNA damage have been observed after strenuous effort, but this response was always less pronounced in well-trained athletes than in nonfit subjects (9, 29, 38).

**Limitations of the study.** All investigations took place during a 2-wk period to eliminate any seasonal influences. However, this short time frame limited the volunteer recruitment, particularly among the combat swimmers and the Naval Pentathlon athletes. Thus the sample size of the individual groups was fairly variable. In addition, albeit we included only male nonsmokers to avoid the impact of sex and/or smoking habits, we cannot rule out selection bias as a confounding factor in our study due to the fact that vulnerable and/or nonfit subjects would of course stay away from activities such as diving and/or extreme endurance.

**Conclusion.** Combat swimmers and UDT divers, i.e., subjects with repetitive and prolonged exposures to HBO over years, and nondiving volunteers both with (Naval Pentathlon athletes) and without endurance training did not show any difference in the incidence of blood cell DNA damage or in antioxidant enzyme activity or capacity. Although combat swimmers showed the most pronounced O2•− formation and increase of DNA damage after ex vivo HBO exposure of isolated lymphocytes, complete removal of DNA damage was at least as rapid as in the other groups.

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**GRANTS**

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