Apoptosis (2009) 14:97–107
DOI 10.1007/s10495-008-0280-z

ORIGINAL PAPER

Hyperbaric oxygen induces apoptosis via a mitochondrial mechanism

Stefan U. Weber · Andreas Koch · Jens Kankeleit · Jens-Christian Schewe · Ullrich Siekmann · Frank Stüber · Andreas Hoeft · Stefan Schröder

Published online: 4 December 2008
© Springer Science+Business Media, LLC 2008

Abstract During therapeutic hyperbaric oxygenation lymphocytes are exposed to high partial pressures of oxygen. This study aimed to analyze the mechanism of apoptosis induction by hyperbaric oxygen. For intervals of 0.5–4 h Jurkat-T-cells were exposed to ambient air or oxygen atmospheres at 1–3 absolute atmospheres. Apoptosis was analyzed by phosphatidylserine externalization, caspase-3 activation and DNA-fragmentation using flow cytometry. Apoptosis was already induced after 30 min of hyperbaric oxygenation (HBO, $P < 0.05$). The death receptor Fas was downregulated. Inhibition of caspase-9 but not caspase-8 blocked apoptosis induction by HBO. Hyperbaric oxygen caused a loss of mitochondrial membrane potential and caspase-9 induction. The mitochondrial pro-survival protein Bcl-2 was upregulated, and antagonizing Bcl-2 function potentiated apoptosis induction by HBO. In conclusion, a single exposure to hyperbaric oxygenation induces lymphocyte apoptosis by a mitochondrial and not a Fas-related mechanism. Regulation of Fas and Bcl-2 may be regarded as protective measures of the cell in response to hyperbaric oxygen.

Keywords Hyperbaric oxygen · Diving · Sepsis · Apoptosis · Lymphocyte · Mitochondrion · Bcl-2 · Fas · Caspase-3

S. U. Weber (✉) · J. Kankeleit · J.-C. Schewe · A. Hoeft
Department of Anesthesiology and Intensive Care Medicine, University of Bonn Medical Center, Sigmund Freud Str. 25, 53105 Bonn, Germany
e-mail: Stefan.weber@ukb.uni-bonn.de

A. Koch
German Naval Medical Institute, Kopperpahler Allee 120, 24119 Kronshagen, Germany

U. Siekmann
Department of Anesthesiology and Intensive Care Medicine, University Hospital of Aachen, Pauwelsstraße 30, 52057 Aachen, Germany

F. Stüber
Department of Anaesthesiology and Pain Therapy, University Hospital Bern "Inselspital", 3010 Bern, Switzerland

S. Schröder
Clinic of Anesthesiology and Intensive Care Medicine, Westküstenklinikum Heide, Esmarch Str. 50, 25746 Heide, Germany

Introduction

Hyperbaric oxygenation (HBO) and diving with rebreathing systems expose individuals to high oxygen concentrations. During HBO the patient is placed in a pressure chamber and is repeatedly exposed to high partial pressures of oxygen [1, 2]. According to the Undersea and Hyperbaric Medical Society indications comprise carbon monoxide poisoning, gas embolism [3], clostridial myonecrosis [4], compartment syndrome and other traumatic ischemia, non-healing wounds [5], necrotizing soft tissue infections and others [2, 6]. Current research also investigates beneficial effects of HBO in the context of sepsis [7–10]. HBO increases the physically solved oxygen in blood multifold resulting in a partial pressure of about 2,200 mmHg and an oxygen concentration of about 27 ml O_2/dl blood at 3 atmospheres absolute (ATA) [2, 11].

This increased oxygen content is known to cause several cellular effects including the inhibition of interferon-γ [12], interleukin-1β and tumor necrosis factor α release [13], a temporary drop in the CD4:CD8 lymphocyte ratio [14], upregulation of the interleukin-2 receptor and downregulation of the $\alpha\beta$ T-cell receptor [15],

induction of DNA damage and compensatory heme oxygenase-1 upregulation [16–20], decrease of lymphocyte proliferation [21] and inhibition of human mammary epithelial proliferation [22]. As an overall effect, at least in rodents, hyperbaric oxygenation appears to be anti-inflammatory since it increases the susceptibility to respiratory infections [23, 24] and delays allograft rejection [25–27]. While hyperbaric oxygenation may inhibit apoptosis in models of ischemic brain injury [28, 29] and ischemic wound healing [30] possibly due to limiting tissue hypoxia, a single exposure to high partial oxygen pressure was observed to enhance apoptosis mouse fibroblasts [31], Jurkat-T-cells, HL-60 cells, murine thymocytes [32] and NCI-H929 [33].

The mechanisms of apoptosis induction by hyperbaric oxygenation involve reactive oxygen species [32]. Moreover, activation of the p38 MAP-kinase pathway was observed [33]. In principal, apoptosis may be initiated via the extrinsic death receptor pathway [34–36] or the mitochondrial intrinsic pathway [37–39]. The extrinsic pathway picks up signals by ligation of death receptors and transmits them to caspase-8 which in turn activates caspase-3 by cleavage [35]. But apoptosis signals may also be generated at the mitochondrial level [40]. There, pro- and antiapoptotic members of the Bcl-2 family of proteins control, the release of cytochrome *c*, APAF-1 and caspase-9, forming the apoptosome [41]. The apoptosome in turn activates caspase-3 [42, 43]. The active effector caspase-3 then degrades multiple targets leading to the common phenotype of the apoptotic cell including DNA-degradation and zeiosis [42]. So far, it is unknown, which pathway transmits the apoptosis signal induced by hyperbaric oxygen.

The aims of our study were to further characterize the apoptotic response to hyperbaric oxygenation in Jurkat-T-cells and to analyze relative contributions of death receptor and mitochondrial pathways.

Materials and methods

Cell culture

The human Jurkat T-cell line (ACC 282), which was established from an acute lymphoblastic leukemia [44], was cultured in 90% RPMI 1640 + 10% FBS + 2 mM L-glutamine (Invitrogen [GIBCO], Karlsruhe, Germany) with 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO_2. Cells were cultured at 0.5–1.5 × 10^6 cells/ml and a split ratio of 1:3 every 3 days was maintained (DSMZ, Braunschweig, Germany). For experiments cells were placed in 24 well plates in fresh medium at a density of 1.0 × 10^6 cells/ml.

Apoptosis induction, caspase and Bcl-2 inhibition

In certain experiments apoptosis was induced by incubation with the activating monoclonal antibody anti-Fas IgM (clone CH11, Immunotech, Marsaille, France) at a final concentration of 200 ng/ml containing protein G. Apoptosis induction with camptothecin (5 µM, Sigma–Aldrich, Hamburg, Germany) for 12 h was used as positive controls.

The inhibitor (Z-IE(OMe)TD(OMe)-FMK (Z-IETD), stock solution 5 mM, Merck, Darmstadt, Germany) was used at 50 µM final concentration to block caspase-8 [45]. To inhibit caspase-9 Z-LE(OMe)HD(OMe)-FMK (Z-LEHD) was applied [46] at 20 µM final concentration. EM20-25 (5-(6-Chloro-2,4-dioxo-1,3,4,10-tetrahydro-2H-9-oxa-1,3-diaza-anthracen-10-yl)-pyrimidine-2,4,6-trione) is a novel cell permeable Bcl-2 ligand that inhibits Bcl-2 functionality [47]. EM20-25 (Merck, Darmstadt, Germany) was dissolved in DMSO and applied at 10 µM 30 min prior to HBO.

Hyperbaric oxygenation

Cells were incubated under iso- or hyperbaric conditions in a temperature controlled pressure chamber (IDE Power-Test 110 Mini-Pressure Chamber, International Dive Equipment, Rosenheim, Germany). Total atmospheric pressure (absolute atmospheres, ATA) was increased over 8 min to reach isopression of 2 or 3 ATA. Isopression was maintained for intervals of 30 min to 4 h followed by an 8 min decompression phase to isobaric pressure. Gas mixtures were used to maintain pH at different pressure levels. At 2 ATA cells were exposed to 97.5% O_2 and 2.5% carbon dioxide (CO_2), while at 3 ATA a mixture of 98.4% O_2 and 1.6% CO_2 were chosen. For control experiments an isobaric atmosphere of 95% oxygen (O_2) and 5% CO_2 or an atmosphere of 21% O_2 and 5% CO_2 were used. In addition, a gas mixture of 7% O_2, 1.6% CO_2 and 91.4% N_2 was chosen to obtain normoxic partial pressure at 3 ATA. If not indicated otherwise, an atmosphere of 21% O_2 and 5% CO_2 at ambient pressure level for 4 h was used as a standard control (termed sham exposure, "sham exp."). During exposure temperature was kept at 37°C. Post exposure cells were transferred to a cell culture incubator and kept in an atmosphere of ambient air containing 5% CO_2 at 37%°C until further analysis.

Measurement of apoptosis

As a marker of early apoptosis phosphatidylserine (PS) externalization was measured by annexin V binding. 5 × 10^5 cells were used per sample. Then they were incubated with annexin-V-fluorescein-isothiocyanate (Becton Dickinson, Heidelberg, Germany) for 10 min at

room temperature in Hepes buffer containing calcium (2,5 mM). Prior to flow cytometric analysis cells were labeled with the DNA-dye 7-actinoaminomycin (7-AAD, Becton Dickinson, Heidelberg, Germany) for the detection of membrane leaks and subjected to data acquisition within 10 min. The percentage of PS^+ but 7-AAD^- population was recorded. To test whether HBO induces membrane permeabilization, in certain experiments, also the percentage of cells positive for 7-AAD (and positive for PS^+) was analyzed.

DNA-fragmentation was monitored as the subdiploid peak in flow cytometry according to Nicoletti et al. [48, 49]. Briefly, 5×10^5 cells were placed for staining in a hypotonic solution of propidium iodide (50 μg/ml) in 0.1% sodium citrate plus 0,1% Triton X-100 at 4°C in the dark overnight before flow cytometric acquisition.

Caspase-3 and -9 activation

For caspase-3 activation measurement 5×10^5 cells were stained per sample. All steps were carried out at 4°C. Cells were washed with phosphate buffered saline (PBS, Sigma–Aldrich, Steinheim, Germany) and fixed with 750 μl PBS containing 4% paraformaldehyde (Sigma–Aldrich, Steinheim, Germany). After fixation, cells were permeabilized with 1 ml PBS containing 0.5% saponin (Sigma–Aldrich, Steinheim, Germany) and 1% bovine serum albumin (BSA; Sigma–Aldrich, Steinheim, Germany). Cells were washed twice with PBS (0.5% saponin/1% BSA). Active caspase-3 was detected by a phycoerythrin (PE)-labeled antibody directed against the active fragment (Becton Dickinson, Heidelberg, Germany). Subsequently, cells were washed twice and resuspended in 250 μl PBS (0.5% saponin/1% BSA) for flow cytometric acquisition.

Caspase-9 activation was analyzed employing a carboxyfluorescein analog of zLEHD-FMK (FAM-zLEHD-FMK Biocarta, San Diego, USA), which irreversibly binds to activated caspase-9. The assay was carried out according to manufacturer's recommendations. Briefly, after washing 5×10^5 cells, they were placed in a total volume of 300 μl PBS. Ten microliter of a 30x solution of FAM-zLEHD-FMK were added and cells were incubated at 37°C for 1 h a cell culture incubator protected from light, followed by two-washing steps and counterstaining with propidium iodide (PI). Until flow cytometric acquisition samples were kept at +4°C. In the flow cytometric analysis only the PI-negative population was analyzed.

Surface receptor and Bcl-2 protein expression

Death receptor and ligands were detected by staining with anti-Fas-PE (Becton Dickinson, Heidelberg, Germany), anti-Fas-ligand (CD178, Euroclone, Wetherby, UK), and anti-TRAIL (CD253, Euroclone, Wetherby, UK). 5×10^5 cells were incubated with anti-Fas-PE for 30 min, after which the sample was fixed with FACS-Lysis-Solution (Becton Dickinson, Heidelberg, Germany), washed twice and stored in the dark at 4°C until analysis. Intracellular Bcl-2 was detected with a specific PE-conjugated monoclonal antibody (Becton Dickinson, Heidelberg, Germany) in parallel to the protocol for caspase-3 staining.

Measurement of mitochondrial membrane potential

Loss of mitochondrial membrane potential was assessed by JC-1 [50] (BD Biosciences, Heidelberg, Germany) staining [51, 52] according to manufacturers instructions. Briefly, 0.5×10^6 cells stained with 10 μg/ml JC-1 for 15 min at 37°C in a CO_2 incubator, followed by two washes. Subsequently cells were subjected to flow cytometric analysis and fluorescence was recorded at 590 and 525 nm. A loss of mitochondrial membrane potential was characterized by a drop in red fluorescence at 590 nm, when JC-1 J-aggregates disintegrate.

Flow cytometry

Stained and fixed cells were measured by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany) and analyzed with CellQuest Pro software (CellQuest Pro, Version 4.0.2, Becton Dickinson, Heidelberg, Germany). Ten thousand events of interest were acquired. Populations of interest were selected as a result of multiple gating using forward (cell size) and sideward (cell granularity) scatter and regions on fluorescence channels 1 or 3. Isotype controls were used for exclusion of background fluorescence; mono-stained controls were used for color-compensation of fluorescence channels. The mean fluorescence intensity (MFI) was recorded. Results were given as linear fluorescence units (LFU) or percentage of positive cells.

Statistics

To evaluate differences between different treatments Student *t*-test was performed for 2 groups and ANOVA analysis was applied for at least 3 groups. To compare time kinetics of different treatments Two-Way ANOVA analysis was used. For post hoc testing the Bonferroni test was employed, when appropriate. Values were given as mean ± standard error of the mean if not indicated differently. Analysis was done with GraphPad for Windows (Version 3.02, San Diego, CA, USA). Experiments were carried out in duplicates, and were reproduced at least twice. Significance was accepted at $P < 0.05$.

Results

HBO induces PS-externalization

Jurkat-T-cells were exposed to a 4 h period of hyperbaric oxygen with a total atmospheric pressure of 3 ATA. PS-externalization was monitored as a marker of early apoptosis for 18 h post exposure. While control cells kept at 21% O_2 ambient atmosphere did not exhibit increased PS-externalization starting from a baseline of 1.86 ± 0.13% (PS^+ 7-AAD^-), HBO significantly increased the percentage of PS-positive starting from 3 h post treatment (5.72 ± 0.28) to 6.25 ± 0.22% ($P < 0.001$) at 6 h, increased to 6.54 ± 0.19% at 12 h and 8.84 ± 0.69% at 18 h (Fig. 1a). HBO did not cause relevant membrane permeabilization when analyzing 7-AAD positivity as a marker of necrosis, Fig. 1a.

Hyperbaric oxygen causes caspase-3 activation

Even more pronounced was the effect of HBO on the activation of caspase-3, the main executioner caspase. While baseline levels remained between 0.43 ± 0.14% and 1.27 ± 0.34% caspase-3 positive cells, caspase-3 activation was induced markedly by HBO treatment. Immediately after exposure caspase-3 activation was still low but increased to 7.8 ± 0.56% at 6 h, 8.93 ± 0.22% at 12 h and 9.03 ± 0.29% at 18 h post exposure (Fig. 1b).

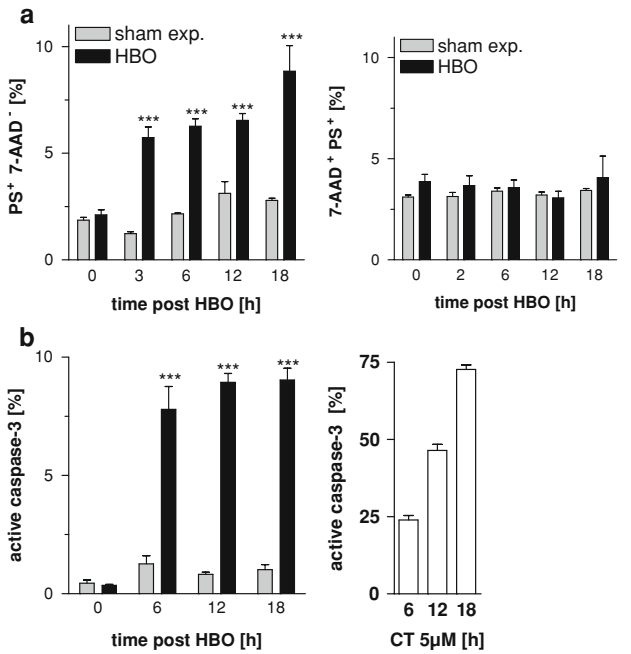

Fig. 1 Hyperbaric oxygen induces apoptosis in a lymphocyte cell line. Jurkat-T-cells were exposed to HBO (3 ATA) for 4 h or to ambient oxygen partial pressure for 4 h (sham exposure, "sham exp."). After HBO cells were kept in a cell culture incubator for 0, 3, 6, 12 and 18 h post exposure. **a** Phosphatidylserine (PS) externalization was measured by Annexin-V binding. The graph shows the percentage of PS^+ 7-AAD^-cells. To evaluate the percentage of necrotic cells also the 7-AAD^+ and PS^+ population was analyzed. **b** Caspase-3 activation was assessed by employing a PE-labeled antibody against activated caspase-3. The left graph displays proportion of the caspase-3^+ subpopulation. Apoptosis activation with camptothecin (CT) 5 µM, was used as a positive control, as displayed in the right graph. Two-way-ANOVA analysis, $n = 3$, $^{***}P < 0.001$

HBO induces DNA-fragmentation

Jurkat-T-cells were exposed to the HBO-conditions as described above. To evaluate DNA-fragmentation, the sub-G1 peak after propidium iodide staining was analyzed 18 h post apoptosis induction by HBO. In these experiments DNA-fragmentation was observed in 20.4 ± 0.643% of cells ($P < 0.001$) as compared to controls kept at 21% O_2 (5.63 ± 0.87%, Fig. 2).

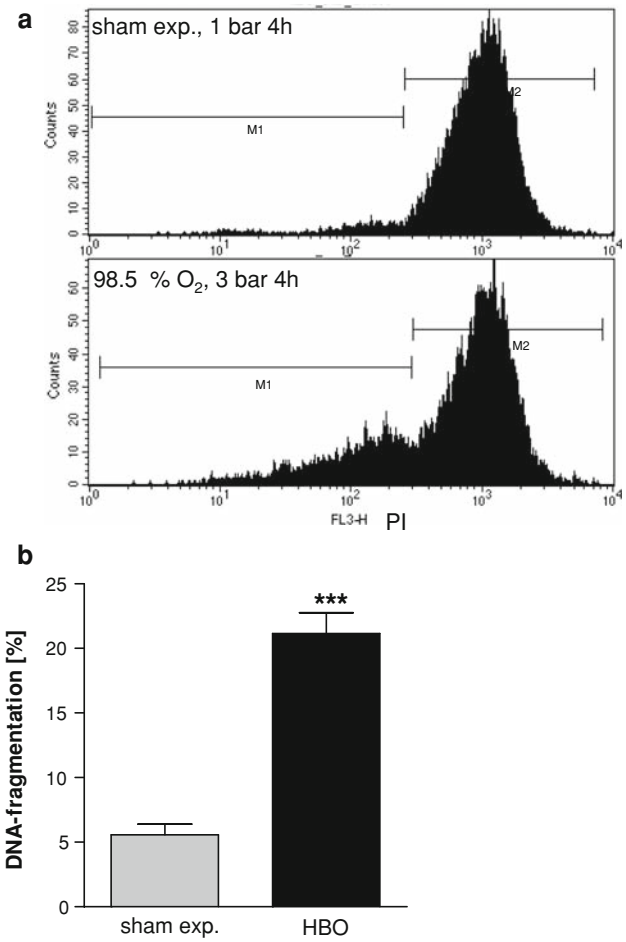

Fig. 2 HBO results in lymphocyte DNA-fragmentation. Jurkat-T-cells were exposed to HBO (3 ATA) or to ambient oxygen partial pressure for 4 h (sham exposure, "sham exp."), after which cells were transferred into an incubator for the following 18 h. DNA-fragmentation was monitored using PE-staining of the subdiploid DNA-peak. **a** Representative histograms displaying sub-G1 fluorescence in region M1. **b** Proportion of cells with a subdiploid DNA-peak. *t*-test, $n = 3$, $^{***}P < 0.001$

Apoptosis increases with a combination of hyperoxia and hyperbaric conditions

To assess the effect of the hyperoxia alone and the influence of the absolute atmospheric pressure, Jurkat-T-cells were exposed to either hyperoxia in a normobaric environment, or hyperbaric oxygenation at 2 or 3 ATA for a time period of 4 h. The CO_2 concentrations were adjusted to keep normal pH. PS-externalization at 12 h post exposure was used as an indicator of apoptosis and the percentage of PS-positive cells (PS^+ 7-AAD^-) before exposure was used as a reference. Hyperoxia (95% O_2 with 5% CO_2) alone did not induce apoptosis. Also, normoxia at 3 ATA (7% O_2 at 3 ATA) did not induce significant apoptosis (1.09-fold, n.s.). Hyperbaric oxygen at 2 ATA showed a small but significant increase (1.4-fold, $P < 0.05$). At 3 ATA apoptosis induction was more pronounced (5.2-fold, $P < 0.001$, Fig. 3a).

Apoptosis increases with exposure time

To evaluate the effect of exposure time Jurkat T-cells were exposed to 3 ATA for time intervals of 0.5–4 h. Wells just placed in the exposure chamber (37°C) and immediately removed without further exposure were used as baseline controls (0 h). PS-externalization (PS^+ 7-AAD^-) was monitored 9 h after treatment. Exposure already for 30 min increased PS-externalization significantly (6.47 ± 0.6 4 vs. 3.73 ± 0.64%, $P < 0.01$). The effect increased further to 4 h with 10.0 ± 0.12% PS-positive cells ($P < 0.01$ vs. 2 h value, Fig. 3b).

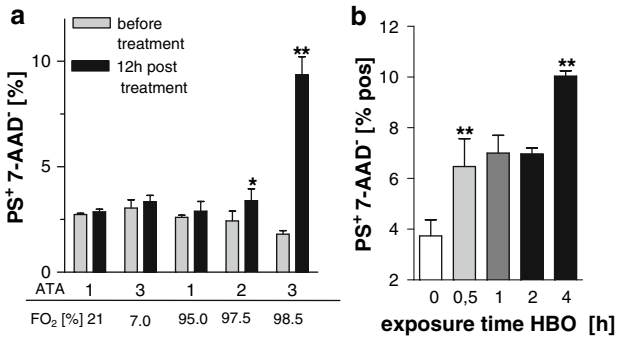

Fig. 3 Induction of apoptosis requires both hyperoxia and increased pressure, and is time dependent. **a** Jurkat-T-cells were exposed to either normoxia in ambient atmosphere (21% O_2, containing 5% CO_2, 1 ATA), 7% O_2, containing 1.6% CO_2 at 3 ATA to achieve normoxia at 3 ATA or hyperoxia (95% O_2 and 5% CO_2 at ambient pressure). HBO was achieved by exposure to 97.5% O_2 and 2.5% CO_2 at 2 ATA, or to 98.4% O_2 and 1.6% CO_2 at 3 ATA. The duration of treatments was 4 h, after which cells were transferred back to the cell culture incubator. PS-externalization (% PS^+ 7-AAD^-) was measured 12 h after exposure and compared to baseline levels before treatment. $n = 3$, Two-way ANOVA, $^*P < 0.05$, $^{**}P < 0.01$. **b** To analyze the effect of the duration of treatment, Jurkat-T-cells were exposed to hyperbaric oxygen at 3 ATA for time intervals ranging from 30 min to 4 h. The externalization of PS was analyzed 12 h post treatment. $n = 3$, ANOVA, $^{**}P < 0.01$

HBO downregulates Fas

To evaluate the effect of hyperbaric oxygen on the regulation of the death receptor and ligand system, we exposed Jurkat-T-cells to HBO at 3 ATA for 4 h and measured the membrane associated expression of Fas, Fas ligand (Fas-L) and TRAIL 12 h after exposure. The baseline expression of TRAIL and Fas-L was low in Jurkat-cells and was not affected significantly by HBO. However, Fas protein was downregulated 32% by exposure to hyperbaric oxygen ($P < 0.01$, Fig. 4a).

Inhibition of caspase-8 does not prevent HBO-induced caspase-3 activation

We aimed to assess the contribution of the death receptor pathway to apoptosis induction by hyperbaric oxygen. Apoptosis in Jurkat-T-cells was induced by HBO (3 ATA, 4 h). Caspase-8 was inhibited 30 min prior to HBO by addition of Z-IETD. Inhibition of caspase-8 failed to inhibit the activation of caspase-3 (Fig. 4). An activating anti-Fas-antibody added prior to HBO potentiated HBO-induced caspase-3 activation threefold. The effect was abrogated by addition of Z-IETD. Notably, also in this part of the experiment only the anti-Fas- but not the HBO-induced proportion caspase-3 activation could be blocked by caspase-8 inhibition (Fig. 5).

HBO causes loss of mitochondrial membrane potential and induces caspase-9 activation

To investigate the involvement of the mitochondrial pathway, depolarization of the mitochondrion was

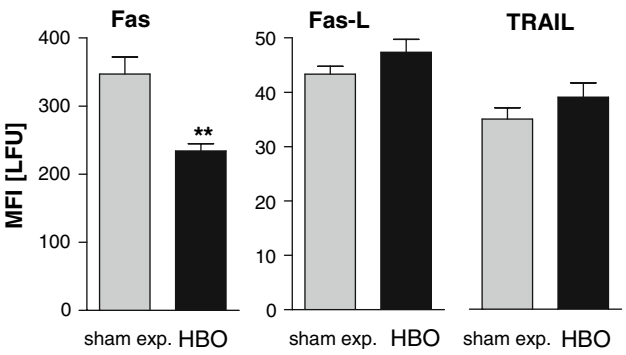

Fig. 4 HBO downregulates Fas expression. Jurkat-T-cells were exposed to HBO (3 ATA) or ambient atmosphere containing 5% CO_2 (sham exposure, "sham exp.") for 4 h. Twelve hours after exposure, the surface expression of Fas, Fas-ligand (Fas-L) and TRAIL was assessed by flow cytometry. $n = 3$, $^{**}P < 0.01$

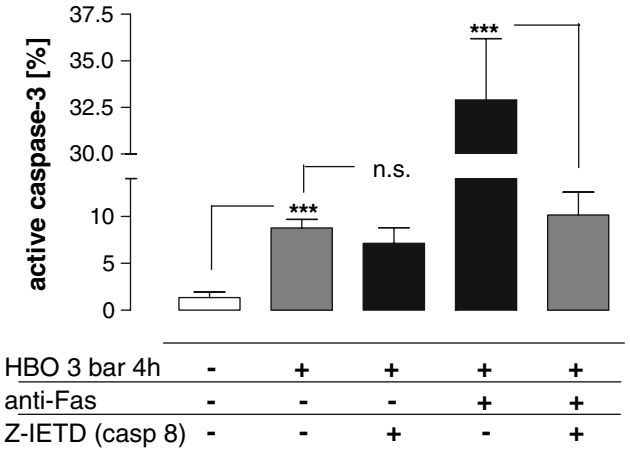

Fig. 5 HBO does not induce apoptosis via caspase-8. To analyze the involvement of the Fas pathway, Jurkat-T-cells were exposed to hyperbaric oxygen at 3 ATA (+) or ambient atmosphere with 5% CO_2 (−). Caspase-3 activation was evaluated by flow cytometry 12 h post exposure. Caspase-8 was inhibited by Z-IETD. Additional apoptosis was induced by an activating anti-Fas antibody (anti-Fas, clone CH11). The Fas-induced proportion of apoptosis could be blocked by Z-IETD, but not the HBO-induced caspase-3 activation. $n = 3$, $***P < 0.001$

analyzed by loading with JC-1. Jurkat T-cells were exposed to HBO (3 ATA, 4 h) and were stained 12 h post exposure. HBO caused a considerable breakdown of mitochondrial membrane potential. 32.3 ± 1.17% cells showed low JC-1 fluorescence, as compared to 4.9 ± 0.35% in cells exposed to 21% O_2 and 5% CO_2 at ambient pressure ($P < 0.01$, Fig. 6a, b). Caspase-9 is activated by mitochondrial release of apoptosome components. In Jurkat-T-cells caspase-9 activation was induced 3 and 6 h after HBO (Fig. 6d).

Inhibition of caspase-9 blocks HBO-induced caspase-3 activation

To assess the apoptotic signal transduction from the mitochondrion to caspase-3 Jurkat-T-cells were pretreated either with the caspase-9 inhibitor Z-LEHD or solvent control. After 30 min cells were either exposed to HBO (3 ATA, 4 h) or a control atmosphere at 1 ATA for 4 h. Twelve hours post exposure caspase-3 activation was measured. Hyperbaric oxygen increased the caspase-3^+ population 4.5-fold as compared to sham exposure ($P < 0.001$). Z-LEHD prevented HBO-induced caspase-3 activation almost completely ($P < 0.001$ vs. HBO, Fig. 6c). The inhibitor alone had no significant effect. Caspase-3 induction by anti-Fas treatment (200 ng/ml containing protein G for 18 h) could not be blocked by 20 μM z-LEHD in our experimental setting (anti-Fas alone 72.85 ± 3.46% induction, with added z-LEHD 70.68 ± 6.70%, $n = 3$).

HBO upregulates Bcl-2 and inhibition of Bcl-2 function potentiates apoptosis

Since the integrity of the outer mitochondrial membrane is guarded by the Bcl-2 family of proteins, the intracellular expression of Bcl-2 or of active-caspase-3 was assessed in Jurkat cells 12 h after exposure to hyperbaric oxygen (3 ATA, 4 h). Overall, a moderate 1.5 ± 0.13-fold upregulation of Bcl-2 could be observed in response to HBO ($P < 0.01$, Fig. 7b). Pretreatment with the Bcl-2 ligand EM20-25 disrupts the interaction of Bcl-2 with Bax. Addition of the ligand without further stimulus did not cause significant caspase-3-activation. HBO led to a 3.68 ± 0.70-fold increase of the caspase-3-active cell population ($P < 0.05$). Pretreatment with EM20-25 clearly potentiated the induction of apoptosis by HBO. Caspase-3-activation was increased 8.53 ± 1.61-fold as compared to sham exposed cells ($P < 0.01$).

Discussion

The study shows that hyperbaric oxygen induces apoptosis in Jurkat-T-cells by a mitochondrial pathway. This has potential implications for HBO treatment regarding immune function. Apoptosis of lymphocytes may contribute to the immunsupressive effects observed in response to treatment with hyperbaric oxygen [12–14]. Local reduction of active lymphocytes may be one of the mechanisms how HBO extends skin allograft survival [25–27]. On the one hand hyperbaric oxygenation may have beneficial effects in the treatment of sepsis, a syndrome with a mortality of 40–60% [53–55]. On the other hand accelerated apoptosis is believed to be a central factor in the emergence of dangerous immunosuppression during sepsis [56–58]. Thus, further studies on HBO-effects during sepsis should include the analysis of lymphocyte apoptosis since HBO, in principle may further temporary immunosuppression.

In our study apoptosis induction was a function of total partial oxygen pressure. An increase in pressure to 3 ATA under normoxia did not result in significant induction of programmed cell death. Apoptosis induction was significant at 2 and 3 ATA. This pressure range is well reached during common HBO treatment schemes. The classical Boerema strategy uses 3 ATA [2]. For grafts, flaps [1] or problem wounds [6] a pressure range between 2.0 and 2.5 ATA is commonly utilized. In time kinetic studies externalization and caspase-3 activation were increased starting from 30 min up to 4 h exposure time. This time frame lies well within the duration of common treatment forms. HBO-treatments range from 30 min to 2 h of exposure to 100% oxygen and are commonly repeated several times per week [1, 2, 6]. Thus, exposure conditions in our study

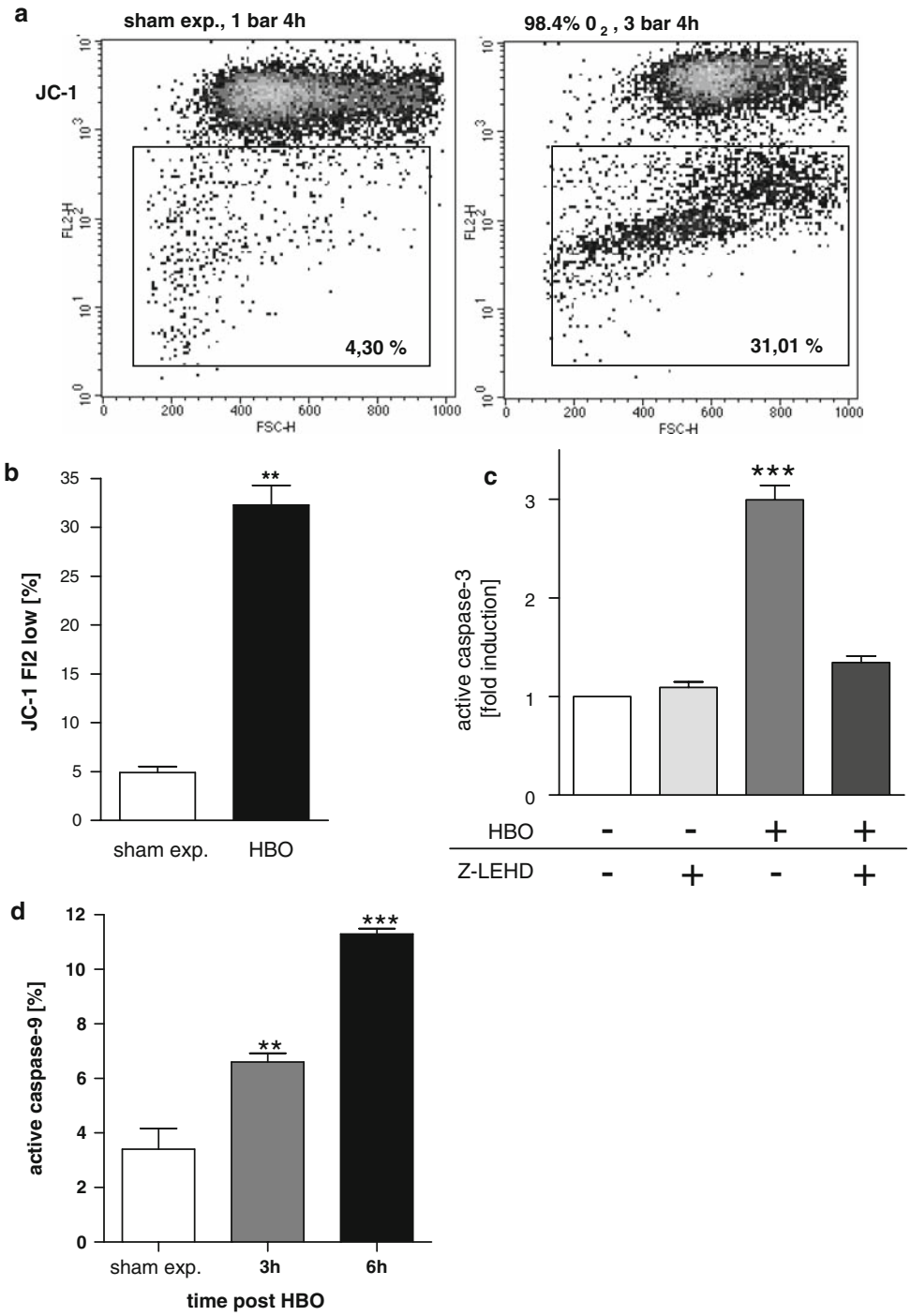

Fig. 6 HBO induces apoptosis via a mitochondrial pathway. Jurkat-cells were placed in an HBO chamber at 3ATA (98.5% O_2) or at 1 ATA (21% O_2, 5% CO_2) as sham controls (sham exposure, "sham exp.") for 4 h. Afterwards, cells were kept in a cell culture incubator for 12 h or the indicated time point until staining with JC-1 or staining for caspase-3/-9 activation, and subsequent flow cytometry. **a** Representative dot plots of JC-1 staining. The region of interest contains cells with a loss of JC-1 fluorescence in channel 2. **b** Percentage of cells bearing low JC-1 fluorescence, $n = 3$, $^{**}P < 0.01$. **c** Prior to HBO cells were pre-treated with either Z-LEHD to inhibit caspase-9 or with a solvent control and caspase-3 activation was measured. Data were expressed as fold induction of active caspase-3 in reference to sham exposed controls (indicated as "−"), $n = 3$ $^{***}P < 0.001$. **d** Caspase-9 activation was evaluated at 3 and 6 h post HBO by staining with FAM-zLEHD-FMK as indicated in "Methods". $n = 3$ $^{**}P < 0.01$, $^{***}P < 0.001$

reflect exposure conditions of lymphocytes in common HBO treatment strategies.

Investigating the death receptor pathway in HBO-induced apoptosis we could show that the main death receptor Fas is clearly downregulated by HBO. No significant regulation of its ligand was observed. The downregulation of Fas may be a protective activity of the cell in response to stress. The apoptotic signal of death receptors is transduced to caspase-8 via FADD [35]. Inhibition of caspase-8 blocks the signal to caspase-3 and inhibits execution of apoptosis [46, 59]. Using the inhibitor Z-IETD [45] we could show that HBO-mediated apoptosis is not transmitted via caspase-8 and thus probably not via the death receptor Fas.

The release of mitochondrial proapoptotic factors often is accompanied by the breakdown of mitochondrial membrane potential [60]. In our experimental setting hyperbaric oxygen induced the loss of mitochondrial membrane

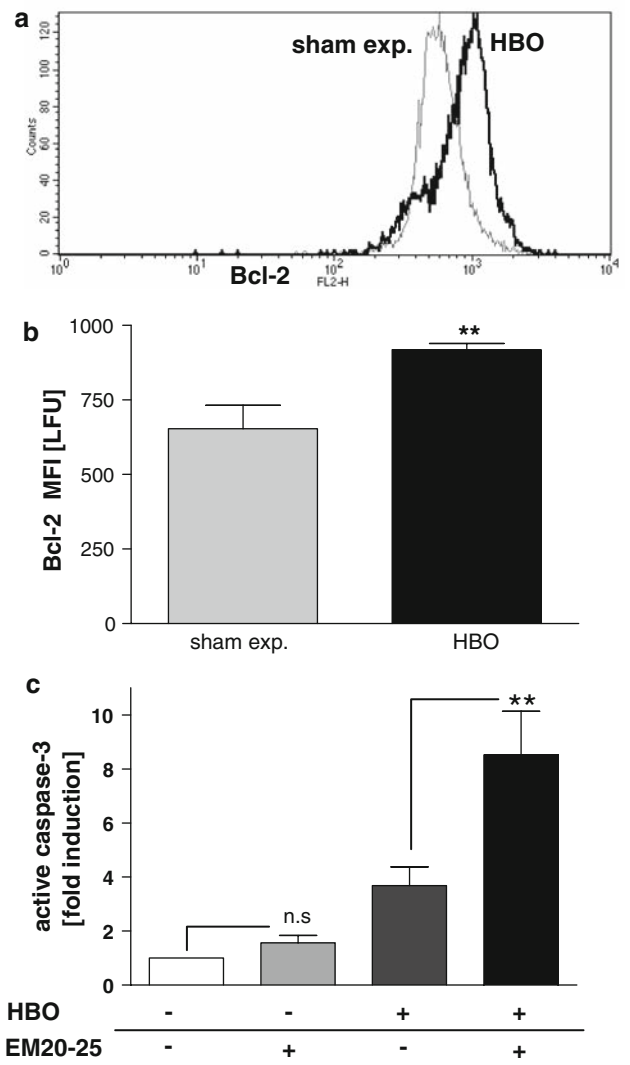

Fig. 7 Jurkat-T-Cells need Bcl-2 to protect against HBO-mediated apoptosis. To assess Bcl-2 expression in the Jurkat-line, cells were exposed to HBO (3 ATA) or ambient atmosphere containing 5% CO_2 (sham exposure, "sham exp.") for 4 h. Twelve hours after exposure cell were stained for intracellular Bcl-2 or active caspse-3 and subjected to flow cytometry. **a** Overlay histogram of a representative experiment. **b** Bcl-2 content of the total population after HBO, expressed as linear fluorescence units. $n = 6$, $^{**}P < 0.01$. **c** Thirty minutes prior to HBO (+) or sham treatment (−) cells were pretreated with the Bcl-2 ligand EM20-25 in order to antagonize Bcl-2 pro-survival activity. Caspase-3 activation was analyzed 12 h post exposure. Data were expressed as fold increase of the cell population with active caspase-3 in reference to sham exposed cells, $n = 3$, $^{**}P < 0.01$

potential. Therefore, we suspect the involvement of a mitochondrial pathway to induce cell death. This is backed by the finding, that apoptosis induction by HBO was sensitive to caspase-9 inhibition.

Since the mitochondrial outer membrane permeabilization is controlled by the Bcl-2 family [61], we next investigated the expression of Bcl-2. For example in sepsis, Bcl-2 downregulation may sensitize lymphocytes to undergo apoptosis [62]. Surprisingly, we found Bcl-2 protein upregulation in the majority of cells, which confers protection of the mitochondrial integrity. Similarly, repeated HBO elevated hippocampal Bcl-2 expression in gerbils [63]. Heme-oxygenase-1 is known to be upregulated in response to HBO in cell cultures [18] as well as in human volunteers [20]. Induction of heme oxygenase-1 protected against HBO-mediated DNA-damage [16–18, 20]. In analogy to the findings regarding heme oxygenase-1 we propose that the upregulation of Bcl-2 may be a protective mechanism of counter regulation in response to proapoptotic stress by hyperbaric oxygen. Therefore, mitochondrial mechanisms independent of Bcl-2 may be involved in apoptosis induction by HBO.

The protective role of Bcl-2 upregulation in response to HBO is further supported by our data employing EM20-25 a small organic Bcl-2 ligand. EM20-25 has been shown to disrupt Bcl-2/Bax interaction without affecting mitochondrial respiration [47]. In our model it did not induce apoptosis per se, but it sensitized Jurkat-T-cells for apoptosis induction by HBO. Thus Bcl-2 appears to be a critical survival factor in the protection against HBO-induced cell death. HBO currently is discussed as a potential additive treatment for leukemia [64]. So far it is unknown, whether human primary lymphocytes also undergo apoptosis in response to HBO in vivo. In this context, the combination of HBO and apoptosis inducing agents may be a promising approach.

In our study we described the effects of a single exposure to HBO. In analogy to the heme-oxygenase-1 mediated upregulation achieved by repetitive HBO [16, 18] an anti-apoptotic gene regulation with increased levels of Bcl-2 and reduced levels of Fas may confer protection against apoptosis after repeated exposure to HBO. This may also help to explain conflicting data from animal experiments where HBO inhibited apoptosis in models of tissue hypoxia [28, 29]. Future experiments need to further analyze the relevance of Blc-2 upregulation and Fas downregulation.

Hyperbaric oxygen causes oxidative stress. The use of a cell culture system with medium may be a limitation of our study [65]. Serum contains antioxidant enzymes and other antioxidants. Thus in vivo, oxidative stress by O_2 may be dampened by the cellular environment. In vitro, cells may be more susceptible to oxidative stress. Although diatomic oxygen molecule is a free radical containing two unpaired electrons it is fairly unreactive [66]. While other reactive gases such as ozone (O_3), nitric oxide (NO_2) or cigarette smoke readily react with many plasma components and cause oxidation/loss of antioxidants, formation of protein carbonyls and reduction of -SH

groups [66, 67], O_2 mainly produces other reactive oxygen species via reactions predominantly located within cells [66]. Ganguly et al. [32] demonstrated that HBO increases oxygen partial pressure not only in the medium but within the cell itself multifold. They also observed increased H_2O_2 production in Jurkat-T-cells. Supplementing the medium with catalase did not inhibit apoptosis, and ascorbic acid probably acted via reducing intracellular H_2O_2 levels. Thus, potentially O_2 effects on cells may not depend on medium components as much as the effects of other reactive gases. Cell culture media contain lower concentrations of ascorbic acid than serum. A lack of ascorbic acid is shown to cause induction of hypoxia-inducible factor (HIF) [68]. HIF-induction inhibits apoptosis in neutrophils and radiation-induced apoptosis in Jurkat-cells [68, 69]. Thus, this effect should not lead to overestimation of apoptosis induced by HBO in cell culture using cell culture medium.

A single exposure to HBO decreased vitamin C and elevated lipid peroxides in human volunteers [70]. Scuba diving at 40 m for 25 min increased lymphocyte H_2O_2 production [71]. A series of 15 HBO treatments increased malondialdehyde and caused reduced activities of superoxide dismutase and catalase [72]. Supplementation with a plant superoxide dismutase prior to HBO inhibited lipid peroxidation as indicated by lowered F2-isoprostane levels [19]. It is well known that mitochondria-generated reactive oxygen species are involved in the release of cytochrome *c* and other pro-apoptotic proteins from the mitochondrion [73]. This is consistent with our finding that the HBO induced apoptosis via the mitochondrion. Oxidative stress caused by HBO may potentially result in the formation of pro-apoptotic substances in the cell or in the medium. Further studies need to address the exact mechanism, how oxidative stress caused by HBO activates the mitochondrial pathway of apoptosis.

Conclusion

Hyperbaric oxygen induces apoptosis in Jurkat-T-cells via a mitochondrial mechanism. A single exposure to hyperbaric oxygen by HBO-therapy or diving with 100%-oxygen using rebreathing systems in principle may induce accelerated lymphocyte cell death. These findings underline the immunomodulatory effect of hyperbaric oxygen. Further human studies should explore this effect in vivo.

Acknowledgments The authors would like to thank Caroline von dem Bussche, Makbule Kobilay and Marcel Schiff for excellent technical assistance. This study was supported by a grant from the German Department of Defense.

References

1. Tibbles PM, Edelsberg JS (1996) Hyperbaric-oxygen therapy. N Engl J Med 334:1642–1648. doi:10.1056/NEJM199606203342506
2. Frey G, Lampl L, Radermacher P, Bock KH (1998) Hyperbaric oxygenation. An area for the anesthetist? Anaesthesist 47:269–289. doi:10.1007/s001010050558
3. Muth CM, Shank ES (2000) Gas embolism. N Engl J Med 342:476–482. doi:10.1056/NEJM200002173420706
4. Leach RM, Rees PJ, Wilmshurst P (1998) Hyperbaric oxygen therapy. BMJ 317:1140–1143
5. Enoch S, Grey JE, Harding KG (2006) ABC of wound healing. Non-surgical and drug treatments. BMJ 332:900–903. doi:10.1136/bmj.332.7546.900
6. Gill AL, Bell CN (2004) Hyperbaric oxygen: its uses, mechanisms of action and outcomes. QJM 97:385–395. doi:10.1093/qjmed/hch074
7. Buras JA, Holt D, Orlow D, Belikoff B, Pavlides S, Reenstra WR (2006) Hyperbaric oxygen protects from sepsis mortality via an interleukin-10-dependent mechanism. Crit Care Med 34:2624–2629. doi:10.1097/01.CCM.0000239438.22758.E0
8. Calzia E, Oter S, Muth CM, Radermacher P (2006) Evolving career of hyperbaric oxygen in sepsis: from augmentation of oxygen delivery to the modulation of the immune response. Crit Care Med 34:2693–2695. doi:10.1097/01.CCM.0000240782.44414.3A
9. Oter S, Radermacher P, Matejovic M (2006) Can (hyperbaric) oxygen turn off the motor of multiorgan dysfunction? Intensive Care Med 32:1694–1696. doi:10.1007/s00134-006-0379-z
10. Muth CM, Radermacher P, Cuzzocrea S (2005) Hyperbaric oxygen and sepsis: time to recognize. Intensive Care Med 31:1150–1152. doi:10.1007/s00134-005-2700-7
11. Treacher DF, Leach RM (1998) Oxygen transport-1. Basic principles. BMJ 317:1302–1306
12. Granowitz EV, Skulsky EJ, Benson RM et al (2002) Exposure to increased pressure or hyperbaric oxygen suppresses interferon-gamma secretion in whole blood cultures of healthy humans. Undersea Hyperb Med 29:216–225
13. Benson RM, Minter LM, Osborne BA, Granowitz EV (2003) Hyperbaric oxygen inhibits stimulus-induced proinflammatory cytokine synthesis by human blood-derived monocyte-macrophages. Clin Exp Immunol 134:57–62. doi:10.1046/j.1365-2249.2003.02248.x
14. Bitterman N, Bitterman H, Kinarty A, Melamed Y, Lahat N (1993) Effect of a single exposure to hyperbaric oxygen on blood mononuclear cells in human subjects. Undersea Hyperb Med 20:197–204
15. Bitterman N, Lahat N, Rosenwald T, Kinarty A, Melamed Y, Bitterman H (1994) Effect of hyperbaric oxygen on tissue distribution of mononuclear cell subsets in the rat. J Appl Physiol 77:2355–2359
16. Groger M, Speit G, Radermacher P, Muth CM (2005) Interaction of hyperbaric oxygen, nitric oxide, and heme oxygenase on DNA strand breaks in vivo. Mutat Res 572:167–172. doi:10.1016/j.mrfmmm.2005.01.015
17. Speit G, Dennog C, Radermacher P, Rothfuss A (2002) Genotoxicity of hyperbaric oxygen. Mutat Res 512:111–119. doi:10.1016/S1383-5742(02)00045-5
18. Rothfuss A, Radermacher P, Speit G (2001) Involvement of heme oxygenase-1 (HO-1) in the adaptive protection of human lymphocytes after hyperbaric oxygen (HBO) treatment. Carcinogenesis 22:1979–1985. doi:10.1093/carcin/22.12.1979
19. Muth CM, Glenz Y, Klaus M, Radermacher P, Speit G, Leverve X (2004) Influence of an orally effective SOD on hyperbaric

oxygen-related cell damage. Free Radic Res 38:927–932. doi:10.1080/10715760412331273197
20. Speit G, Dennog C, Eichhorn U, Rothfuss A, Kaina B (2000) Induction of heme oxygenase-1 and adaptive protection against the induction of DNA damage after hyperbaric oxygen treatment. Carcinogenesis 21:1795–1799. doi:10.1093/carcin/21.10.1795
21. Gadd MA, McClellan DS, Neuman TS, Hansbrough JF (1990) Effect of hyperbaric oxygen on murine neutrophil and T-lymphocyte functions. Crit Care Med 18:974–979. doi:10.1097/00003246-199009000-00014
22. Granowitz EV, Tonomura N, Benson RM et al (2005) Hyperbaric oxygen inhibits benign and malignant human mammary epithelial cell proliferation. Anticancer Res 25:3833–3842
23. Won WD, Ross HC, Deig EF (1976) Influence of cold or hyperbaric helium-oxygen environments on mouse response to a respiratory viral infection. Aviat Space Environ Med 47:704–707
24. Won WD, Ross HC (1975) Catecholamine and phagocytic responses in infected mice exposed to hyperbaric helium-oxygen atmospheres. Aviat Space Environ Med 46:191–193
25. Jacobs BB, Thuning CA, Sacksteder MR, Warren J (1979) Extended skin allograft survival in mice during prolonged exposure to hyperbaric oxygen. Transplantation 28:70–72. doi:10.1097/00007890-197907000-00017
26. Erdmann D, Roth AC, Hussmann J et al (1995) Skin allograft rejection and hyperbaric oxygen treatment in immune-histoincompatible mice. Undersea Hyperb Med 22:395–399
27. Erdmann D, Roth AC, Hussmann J et al (1996) Hyperbaric oxygen and cyclosporine as a combined treatment regimen to prevent skin allograft rejection in immunohistoincompatible mice. Ann Plast Surg 36:304–308. doi:10.1097/00000637-199603000-00013
28. Yin D, Zhou C, Kusaka I et al (2003) Inhibition of apoptosis by hyperbaric oxygen in a rat focal cerebral ischemic model. J Cereb Blood Flow Metab 23:855–864. doi:10.1097/01.WCB.0000073946.29308.55
29. Ostrowski RP, Colohan AR, Zhang JH (2005) Mechanisms of hyperbaric oxygen-induced neuroprotection in a rat model of subarachnoid hemorrhage. J Cereb Blood Flow Metab 25:554–571. doi:10.1038/sj.jcbfm.9600048
30. Zhang Q, Chang Q, Cox RA, Gong X, Gould LJ (2008) Hyperbaric Oxygen attenuates apoptosis and decreases inflammation in an ischemic wound model. J Invest Dermatol 128(8):2102–2112
31. Conconi MT, Baiguera S, Guidolin D et al (2003) Effects of hyperbaric oxygen on proliferative and apoptotic activities and reactive oxygen species generation in mouse fibroblast 3T3/J2 cell line. J Investig Med 51:227–232. doi:10.2310/6650.2003.39201
32. Ganguly BJ, Tonomura N, Benson RM, Osborne BA, Granowitz EV (2002) Hyperbaric oxygen enhances apoptosis in hematopoietic cells. Apoptosis 7:499–510. doi:10.1023/A:1020686908831
33. Chen YC, Chen SY, Ho PS et al (2007) Apoptosis of T-leukemia and B-myeloma cancer cells induced by hyperbaric oxygen increased phosphorylation of p38 MAPK. Leuk Res 31:805–815. doi:10.1016/j.leukres.2006.09.016
34. Green DR, Ferguson TA (2001) The role of Fas ligand in immune privilege. Nat Rev Mol Cell Biol 2:917–924. doi:10.1038/35103104
35. Krammer PH (2000) CD95's deadly mission in the immune system. Nature 407:789–795. doi:10.1038/35037728
36. Wajant H (2002) The Fas signaling pathway: more than a paradigm. Science 296:1635–1636. doi:10.1126/science.1071553
37. Zamzami N, Kroemer G (2001) The mitochondrion in apoptosis: how Pandora's box opens. Nat Rev Mol Cell Biol 2:67–71. doi:10.1038/35048073
38. Martinou JC, Green DR (2001) Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol 2:63–67. doi:10.1038/35048069
39. Willis SN, Adams JM (2005) Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol 17:617–625. doi:10.1016/j.ceb.2005.10.001
40. Rasola A, Bernardi P (2007) The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. Apoptosis 12:815–833. doi:10.1007/s10495-007-0723-y
41. Kim HE, Du F, Fang M, Wang X (2005) Formation of apoptosome is initiated by cytochrome *c*-induced dATP hydrolysis and subsequent nucleotide exchange on Apaf-1. Proc Natl Acad Sci USA 102:17545–17550. doi:10.1073/pnas.0507900102
42. Hengartner MO (2000) The biochemistry of apoptosis. Nature 407:770–776. doi:10.1038/35037710
43. Nicholson DW, Ali A, Thornberry NA et al (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376:37–43. doi:10.1038/376037a0
44. Schneider U, Schwenk HU, Bornkamm G (1977) Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. Int J Cancer 19:621–626. doi:10.1002/ijc.2910190505
45. Martin DA, Siegel RM, Zheng L, Lenardo MJ (1998) Membrane oligomerization and cleavage activates the caspase-8 (FLICE/MACHalpha1) death signal. J Biol Chem 273:4345–4349. doi:10.1074/jbc.273.8.4345
46. Thornberry NA, Lazebnik Y (1998) Caspases: enemies within. Science 281:1312–1316. doi:10.1126/science.281.5381.1312
47. Milanesi E, Costantini P, Gambalunga A et al (2006) The mitochondrial effects of small organic ligands of BCL-2: sensitization of BCL-2-overexpressing cells to apoptosis by a pyrimidine-2, 4, 6-trione derivative. J Biol Chem 281:10066–10072. doi:10.1074/jbc.M513708200
48. Ettorre A, Andreassi M, Anselmi C, Neri P, Andreassi L, Di Stefano A (2003) Involvement of oxidative stress in apoptosis induced by a mixture of isothiazolinones in normal human keratinocytes. J Invest Dermatol 121:328–336. doi:10.1046/j.1523-1747.2003.12360.x
49. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139:271–279. doi:10.1016/0022-1759(91)90198-O
50. Smiley ST, Reers M, Mottola-Hartshorn C et al (1991) Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. Proc Natl Acad Sci USA 88:3671–3675. doi:10.1073/pnas.88.9.3671
51. Reers M, Smiley ST, Mottola-Hartshorn C, Chen A, Lin M, Chen LB (1995) Mitochondrial membrane potential monitored by JC-1 dye. Methods Enzymol 260:406–417. doi:10.1016/0076-6879(95)60154-6
52. Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C (1993) A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun 197:40–45. doi:10.1006/bbrc.1993.2438
53. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR (2001) Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med 29:1303–1310. doi:10.1097/00003246-200107000-00002
54. Brun-Buisson C (2000) The epidemiology of the systemic inflammatory response. Intensive Care Med 26(Suppl 1):S64–S74. doi:10.1007/s001340051121
55. Brunkhorst FM (2006) Epidemiology, economy and practice—results of the German study on prevalence by the competence

network sepsis (SepNet) [Epidemiologie, Okonomie und Praxis—Ergebnisse der deutschen Pravalenzstudie des Kompetenznetzwerkes Sepsis (SepNet)]. Anasthesiol Intensivmed Notfallmed Schmerzther 41(1):43–44
56. Hotchkiss RS, Chang KC, Swanson PE et al (2000) Caspase inhibitors improve survival in sepsis: a critical role of the lymphocyte. Nat Immunol 1:496–501. doi:10.1038/82741
57. Hotchkiss RS, Karl IE (2003) The pathophysiology and treatment of sepsis. N Engl J Med 348:138–150. doi:10.1056/NEJMra021333
58. Perl M, Chung CS, Ayala A (2005) Apoptosis. Crit Care Med 33:S526–S529. doi:10.1097/01.CCM.0000185499.28006.4C
59. Salmena L, Lemmers B, Hakem A et al (2003) Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. Genes Dev 17(7):883–895 Epub 2003 Mar 21
60. Green DR, Kroemer G (2004) The pathophysiology of mitochondrial cell death. Science 305:626–629. doi:10.1126/science.1099320
61. Green DR, Kroemer G (2005) Pharmacological manipulation of cell death: clinical applications in sight? J Clin Invest 115:2610–2617. doi:10.1172/JCI26321
62. Hotchkiss RS, Osmon SB, Chang KC, Wagner TH, Coopersmith CM, Karl IE (2005) Accelerated lymphocyte death in sepsis occurs by both the death receptor and mitochondrial pathways. J Immunol 174:5110–5118
63. Wada K, Miyazawa T, Nomura N et al (2000) Mn-SOD and Bcl-2 expression after repeated hyperbaric oxygenation. Acta Neurochir Suppl (Wien) 76:285–290
64. Tonomura N, Granowitz EV (2007) Hyperbaric oxygen: a potential new therapy for leukemia? Leuk Res 31:745–746. doi:10.1016/j.leukres.2006.11.020
65. Halliwell B (2003) Oxidative stress in cell culture: an underappreciated problem? FEBS Lett 540:3–6. doi:10.1016/S0014-5793(03)00235-7
66. Halliwell B, Cross CE (1994) Oxygen-derived species: their relation to human disease and environmental stress. Environ Health Perspect 102(Suppl 10):5–12. doi:10.2307/3432205
67. Cross CE, Valacchi G, Schock B et al (2002) Environmental oxidant pollutant effects on biologic systems: a focus on micronutrient antioxidant-oxidant interactions. Am J Respir Crit Care Med 166:S44–S50. doi:10.1164/rccm.2206015
68. Vissers MC, Wilkie RP (2007) Ascorbate deficiency results in impaired neutrophil apoptosis and clearance and is associated with up-regulation of hypoxia-inducible factor 1alpha. J Leukoc Biol 81:1236–1244. doi:10.1189/jlb.0806541
69. Weinmann M, Marini P, Jendrossek V et al (2004) Influence of hypoxia on TRAIL-induced apoptosis in tumor cells. Int J Radiat Oncol Biol Phys 58:386–396. doi:10.1016/j.ijrobp.2003.09.052
70. Bader N, Bosy-Westphal A, Koch A et al (2007) Effect of hyperbaric oxygen and vitamin C and E supplementation on biomarkers of oxidative stress in healthy men. Br J Nutr 98:826–833. doi:10.1017/S0007114507744380
71. Ferrer MD, Sureda A, Batle JM, Tauler P, Tur JA, Pons A (2007) Scuba diving enhances endogenous antioxidant defenses in lymphocytes and neutrophils. Free Radic Res 41:274–281. doi:10.1080/10715760601080371
72. Benedetti S, Lamorgese A, Piersantelli M, Pagliarani S, Benvenuti F, Canestrari F (2004) Oxidative stress and antioxidant status in patients undergoing prolonged exposure to hyperbaric oxygen. Clin Biochem 37:312–317. doi:10.1016/j.clinbiochem.2003.12.001
73. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B (2007) Mitochondria, oxidative stress and cell death. Apoptosis 12:913–922. doi:10.1007/s10495-007-0756-2