



Effects of hyperbaric oxygen treatment on antimicrobial function and apoptosis of differentiated HL-60 (neutrophil-like) cells

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ABSTRACT

Aims: Neutrophil apoptosis is important in the resolution of inflammation in chronic wounds. Hyperbaric oxygen (HBO) therapy, an intermittent inhalation of 100% oxygen at greater than atmospheric pressure, appears to be an effective treatment for chronic wounds. The aim was to use HL-60 cells differentiated using all-trans retinoic acid (ATRA) (neutrophil-like cells) to test the hypothesis that an HBO-induced increase in antimicrobial activity might lead to an increase in apoptosis, thereby contributing to neutrophil clearance from chronic wounds.

Main methods: ATRA differentiated HL-60 cells, an *in vitro* neutrophil model, were used to test the effects of normoxia, hypoxia (5% O₂), hyperoxia (95% O₂), hyperbaric normoxia (pressure) (8.8% O₂ at 2.4 ATA) and HBO (97.9% O₂ at 2.4 ATA) on antimicrobial function [NBT staining, superoxide and H₂O₂ production, and phagocytosis activity] and apoptosis (caspase 3/7 activity and morphological changes observed using SYBR Safe staining).

Key findings: A single 90 min HBO exposure caused an increase in the respiratory burst activity of neutrophil-like cells post exposure. Phagocytosis of *Staphylococcus aureus* was also increased. HBO pre-treatment had a pro-apoptotic effect, increasing caspase 3/7 activity and causing morphological changes associated with apoptosis.

Significance: The potential detrimental effect of enhanced antimicrobial activity induced by HBO may be offset by enhanced apoptosis. Both hyperoxia and pressure alone seemed to contribute to the HBO-induced increases in antimicrobial activity and apoptosis, although there was no consistent pattern. These data contribute to explaining the effectiveness of HBO in the treatment of chronic wounds.

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Introduction

The main function of neutrophils is the destruction and clearance of microorganisms at wound sites; this is achieved by two mechanisms: (a) production of reactive oxygen species (ROS) via the 'respiratory' burst; and (b) release of antimicrobial peptides and proteins into phagosomes (degranulation) after engulfment of bacteria (Segal, 2005). After neutrophils complete their task, their clearance from injured tissue, via apoptosis, is essential for the resolution of inflammation (Simon, 2003). However, a characteristic of chronic wounds, where there is prolonged inflammation, is excessive influx and survival of neutrophils in the wound site, promoted by the hypoxic conditions there (Bjarnsholt et al., 2008). This neutrophil accumulation causes extensive damage to tissue via the release of cytotoxic enzymes such as collagenase and elastase, as well as ROS and inflammatory cytokines, thereby exacerbating the problem.

Hyperbaric oxygen (HBO) therapy is the inhalation of 100% oxygen at pressures greater than one atmosphere (1 ATA), repeated once or

twice daily for 60–90 min (Gill and Bell, 2004). HBO leads to an increase in dissolved oxygen in the blood and tissues, and it is commonly used as an adjuvant therapy for inflammatory conditions with an involvement of hypoxia, including chronic wounds. There is some understanding of mechanisms by which HBO may have beneficial effects (Broussard, 2004), but there are still questions relating to mechanisms by which HBO aids the resolution of inflammation, particularly relating to how HBO affects the specific types of inflammatory cell.

The promyelocytic leukaemia cell line HL-60 contains cells with heterogeneous morphology resembling myeloblasts and promyelocytes (Gallagher et al., 1979). However, they can be differentiated by the addition of DMSO (Collins et al., 1979) or retinoic acid (either all *trans* or 9-*cis*) (Verstuyf et al., 1995) to the growth medium, such that the majority of cells come to resemble neutrophils both morphologically and functionally. While there has been some previous work on the effects of HBO on undifferentiated HL-60 cells (e.g. McIntyre et al., 1997), this has not been extended to differentiated HL-60 cells. Hence, the aim of current study was to use HL-60 cells differentiated using all-trans retinoic acid (neutrophil-like cells) as a model to test the hypothesis that an HBO-induced increase in neutrophil antimicrobial activity might lead to an increase in neutrophil apoptosis, thereby contributing

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to their clearance from chronic wounds. The neutrophil-like cells were exposed to HBO (hyperbaric hyperoxia) as well as normobaric hyperoxia, hyperbaric normoxia and normobaric hypoxia and a range of end-points examined post exposure including antimicrobial defences, phagocytosis, and apoptotic cell death.

Materials and methods

All reagents were from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Cell culture

HL-60 cells (passage 6, acquired from ECCAC [98070106]) were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin in vented 75 cm² culture flasks (Bibby Sterilin, UK), and maintained at 37 °C under 5% CO₂. Passages 20–45 were used for experiments. Differentiation of HL-60 cells was induced by incubation 2.5 × 10⁵ cells ml⁻¹ with all-*trans* retinoic acid (ATRA, 1 µM) for 5 days (Verstuyf et al., 1995). The viability of differentiated cells was 80–85%, as determined by trypan blue exclusion.

Hyperbaric oxygenation

Neutrophil-like cells (10⁶ ml⁻¹) in 96-well plates were exposed to hyperbaric oxygen at 20 °C in custom-made pressure chambers (Kendall, 2010) loaned and maintained by DDRC (Plymouth, UK) on behalf of the British Hyperbaric Association. Hyperbaric oxygen conditions were obtained by flushing the chambers for 4 min at 3 l min⁻¹ with 97.9% O₂, 2.1% CO₂, and then pressurizing to 2.4 ATA over 2 min (equivalent to 5% CO₂ at 1 ATA). To investigate the pressure effects, cells were pressurized with 8.8% O₂, 2.1% CO₂ and 89.1% N₂ at 2.4 ATA (equivalent to 21% O₂ at 1 ATA). For normobaric hyperoxia and normobaric hypoxia treatments, cells were exposed to 95% O₂, 5% CO₂ and 5% O₂, 5% CO₂, respectively, in gas-tight plastic boxes. Control cell cultures for each experiment were placed in similar boxes under 21% O₂, 5% CO₂. In all cases the remaining gas was N₂. Gas mixes were prepared by DDRC. Treatments were for 90 min, after which the chambers were decompressed over 8–10 min, the cells were removed and washed by centrifugation for 5 min at 400 g followed by re-suspension to 1–2 × 10⁶ cells ml⁻¹ in a medium appropriate for use in the following assays.

Superoxide production

Superoxide production was measured using reduction of ferricytochrome *c* (Edwards, 1996). Ferricytochrome *c* (5 µl of 2 mM, Sigma C-7752) was added to wells in a 96-well plate followed by 50 µl of 10⁶ cells ml⁻¹ in HBSS, 145 µl HBSS and 10 µl of 100 ng ml⁻¹ phorbol myristate acetate (PMA). PMA solution was prepared by dilution of a 1 mg ml⁻¹ in DMSO with HBSS. After adding the stimulus the absorbance at 550 nm was monitored in a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA) for 45 min at 37 °C. Two controls were used, one where the stimulus was replaced with 10 µl HBSS, and the other where it was replaced with 2 µl of 3000 U ml⁻¹ superoxide dismutase (Sigma S-3409) and 8 µl HBSS. Cytochrome *c* reduction was calculated using an extinction coefficient of 21.1 mM⁻¹ cm⁻¹ and a path length of 0.66 cm.

NBT reduction

Nitroblue tetrazolium (NBT) reduction was also used to assess respiratory burst activity (Verstuyf et al., 1995). Cells (100 µl of 10⁶ ml⁻¹ in RPMI-1640) were mixed with 100 µl of 100 ng ml⁻¹ PMA and 100 µl of 5 mg ml⁻¹ NBT, and incubated for 30 min at 37 °C. After incubation the

percentage of cells containing blue–black formazan deposits was determined by microscopy following preparation of cytospin slides (400 g for 5 min in a Shandon Cytospin centrifuge; > 200 cells assessed per slide).

Measurement of hydrogen peroxide

A horseradish peroxidase (HRP)-linked assay was used to measure the production of H₂O₂ by neutrophil-like cells, using homovanillic acid, the oxidation of which by H₂O₂ yields a fluorescent product (Baggiolini et al., 1986). Briefly, 100 µl of differentiated HL-60 cell suspension in HBSS, containing 10⁶ cells ml⁻¹, was added to each well of a 96-well plate. Then 100 µl of HBSS containing 200 µM homovanillic acid, 10 U ml⁻¹ HRP and 100 ng ml⁻¹ PMA was added per well, and the fluorescence measured after 120 min at 37 °C using a CytoFluor II fluorescence microplate reader (PerSeptive Biosystems, Framingham, USA) with excitation and emission wavelengths of 320 nm and 420 nm, respectively. A standard curve (0–100 µM H₂O₂) was used to determine the H₂O₂ concentration in each well.

Phagocytosis activity

Cells (10⁶ ml⁻¹ in PBS) were incubated with *Staphylococcus aureus* NCIMB 6571 cells (10⁷ cells ml⁻¹ in PBS) for 30 min at 37 °C (Campbell et al., 2001). Phagocytosis was stopped with cold PBS followed by centrifugation at 400 g for 5 min. After another wash with cold PBS, cells were fixed in methanol (30 min) and stained with Giemsa stain. Cells (>500) were examined by light microscopy, and phagocytic index calculated (Campbell et al., 2001).

Myeloperoxidase

Myeloperoxidase (MPO) activity was measured as described by Dypbukt et al. (2005). Cells were re-suspended at 2 × 10⁶ ml⁻¹ in 10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl and 10 mM KCl. Taurine was then added (25 µl of 5 mM to 100 µl of cells) and pre-incubated for 15 min at 37 °C. The stimulus to release MPO, 125 µl of 100 ng ml⁻¹ PMA, was then added and the mixture incubated for 30 min at 37 °C. Finally catalase (10 µl, containing 0.4 U) was added and the mixture was centrifuged at 13,000 g for 2 min. Taurine chloramine was detected by mixing of supernatant or standard (200 µl) with 50 µl of developing reagent (2 mM TMB in 400 mM Na-acetate buffer, pH 5.4, containing 10% dimethylformamide and 100 µM NaI). The absorbance was recorded at 650 nm after 5 min.

Cell viability

Neutrophil-like cells (50 µl of 10⁶ cells ml⁻¹ in supplemented RPMI-1640) were mixed with 0.4% trypan blue (50 µl) and incubated for 5 min. Total and non-viable (i.e. trypan blue stained) cells were counted using a haemocytometer. For assessment of viability using MTT, cells were resuspended in RPMI-1640 at 10⁶ cells ml⁻¹. Cell suspension (100 µl) was placed in 96-well plates and then 10 µl of MTT (5 mg/ml in PBS) was added to each well and incubated for 2–3 h at 37 °C. Finally, DMSO (100 µl) was added to each well and incubated for 20–30 min to solubilise any formazan product. Plates were read on a VersaMax plate reader (Molecular Devices, Sunnyvale, CA, USA) at 540 nm.

Apoptosis

After oxygen treatment, washed cells were incubated at 37 °C for 3 h with or without TNF-α (100 µl of 106 cells ml⁻¹ in PBS plus 10 µl of 100 ng ml⁻¹ TNF-α [Insight Biotechnology, Wembley, UK]). Caspase 3/7 activity was measured using the Apo-ONE® homogenous caspase 3/7 assay (Promega, Southampton, UK), which uses a

fluorometric substrate, Z-DEVD-R110. Fifty microliters of cell suspension was incubated with 50 μ l of the fluorometric substrate in a 96-well plate for 18 h at 20 °C, after which fluorescence was measured using a CytoFluor II plate reader (excitation wavelength, 490 nm; emission wavelength, 525 nm).

Apoptosis was also quantified using the fluorescent DNA-binding dye SYBR Safe. Slides of 10^5 cells in 100 μ l of supplemented RPMI-1640 were prepared using a Shandon Cytospin centrifuge (400 g for 5 min) and stained using SYBR Safe. Morphological change characteristic of apoptosis (Martin et al., 1990) was assessed microscopically using a $\times 100$ objective. Triplicate slides were prepared for each condition and > 500 cells/slide were examined.

Statistics

Statistical analysis was carried out using Statgraphics Centurion XVI (StatPoint Technologies, Inc.). One-way ANOVA or Kruskal–Wallis were used as appropriate. After ANOVA, multiple range tests (Tukey) were used to determine significant differences; after Kruskal–Wallis significant differences were determined by examining median notch box and whisker plots. $P < 0.05$ is considered significant throughout.

Results

Effects of HBO on antimicrobial activity of neutrophil-like cells

HL-60 cells differentiated using all-*trans* retinoic acid (ATRA) were exposed to hyperbaric hyperoxia (97.9% O₂, 2.1% CO₂ at 2.4 ATA), hyperbaric normoxia (8.75% O₂ at 2.4 ATA), normobaric hyperoxia (95% O₂, 5% CO₂ at 1 ATA), normobaric normoxia (21% O₂, 5% CO₂ at 1 ATA) and normobaric hypoxia (5% O₂, 5% CO₂ at 1 ATA) for 90 min at 20 °C. After exposure, respiratory burst activity was assessed by measuring superoxide (O₂⁻) production using both cytochrome *c* reduction (Fig. 1A) and the percentage of nitroblue tetrazolium (NBT) staining cells (Fig. 1B), or by measuring the H₂O₂ production (Fig. 2) using the horseradish peroxidase/homovanillic acid (HRP/HVA) method. In all cases, phorbol myristic acid (PMA) was used to stimulate the neutrophil-like cells.

Addition of PMA to ATRA-treated HL-60 cells caused an increased production of O₂⁻ indicating a successful differentiation to neutrophil-like cells (Fig. 1A). Exposure to HBO before PMA addition caused a significant increase in O₂⁻ production (Fig. 1A) and an increase in the percentage of O₂⁻ producing cells (Fig. 1B), compared to the control when PMA was added post exposure. Prior exposure to hypoxia had the opposite effect on O₂⁻ production. Pressure or hyperoxia alone did not lead to a significant increase in PMA-induced O₂⁻ production, but did cause significant increases in the percentage of cells staining with NBT. Significantly higher H₂O₂ production by PMA-stimulated neutrophil-like cells was observed compared to unstimulated cells (data not shown). Post HBO there was a significant increase in H₂O₂ release from PMA-stimulated cells; the same was also seen post exposure to pressure alone, whereas PMA-stimulated H₂O₂ release was significantly lower post exposure to hypoxia (Fig. 2). It should be emphasised that the cells were washed after oxygen pre-treatment, and before the addition of PMA, so the H₂O₂ measured had not accumulated during the oxygen pre-treatment.

Phagocytic activity with *S. aureus* NCIMB 6571 was measured in ATRA-differentiated HL-60 cells. There was a significant increase in the phagocytic index post HBO (Fig. 3). However, a greater increase was seen following hyperoxia alone, while pressure and hypoxia pre-treatments had no significant effect on the phagocytic index. Degranulation of the neutrophil-like cells was assessed using myeloperoxidase (MPO) release. There were significant increases in PMA-induced MPO release following HBO, hyperoxia and pressure alone (Fig. 4), with the greatest increases with pressure and HBO.

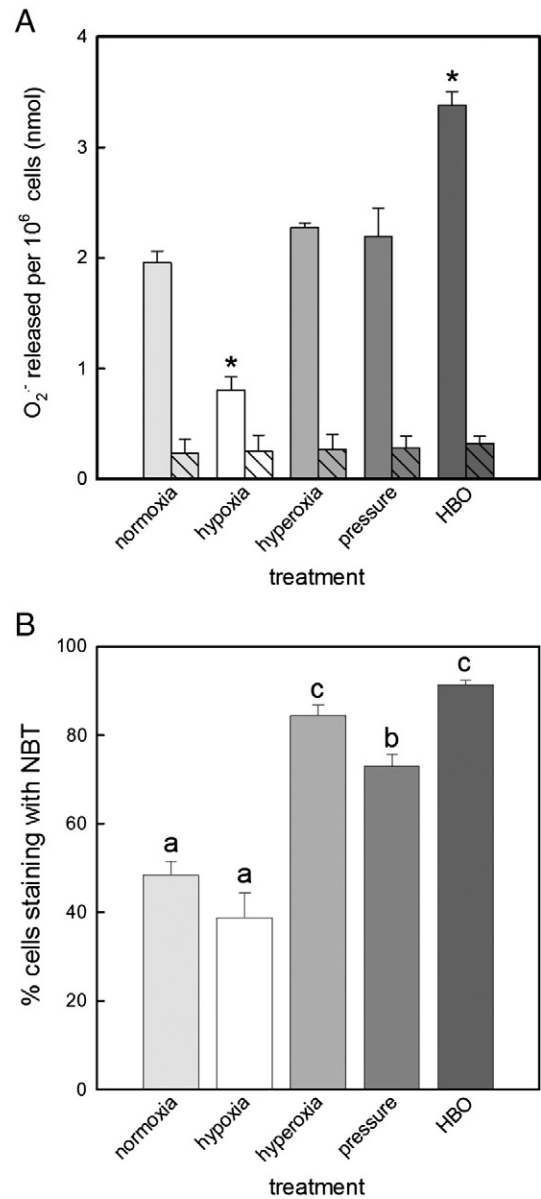


Fig. 1. Effects of oxygen treatments on O₂⁻ production by neutrophil-like cells. ATRA-differentiated HL-60 cells were exposed to a range of oxygen conditions for 90 min (see Materials and Methods), after which they were washed. After PMA treatment (hatched bars indicate absence of PMA), O₂⁻ production was detected using cytochrome *c* (A) and the % NBT staining cells was determined (B). Data are means \pm SEM for three separate experiments (passage numbers 20, 24 and 28) with triplicate measurements ($n = 3$). * significant difference versus normoxia; bars with the same letter are not significantly different (1-way ANOVA, $P = 0.0007$ and < 0.00005 for A and B, respectively).

Effects of HBO on cell death in neutrophil-like cells

Hyperoxia, pressure and HBO all caused similar significant decreases in cell viability assessed using trypan blue staining (Fig. 5A); results using MTT reduction (based on reduction of MTT by intracellular enzymes to an insoluble formazan) were similar (Fig. 5B). Pre-exposure to hypoxia had no significant effect on the viability of the neutrophil-like cells. Pre-exposure to hyperoxia, pressure and HBO produced a significant increase in caspase 3/7 activity in cells treated with TNF- α to induce apoptosis whereas hypoxia caused a decrease (Fig. 6). Although overall levels of activity were lower, a similar pattern was seen in the absence of TNF- α , with significantly higher levels post exposure to pressure and HBO. Apoptosis was also assessed using SYBR Safe staining. Significant increases in cells showing morphological changes

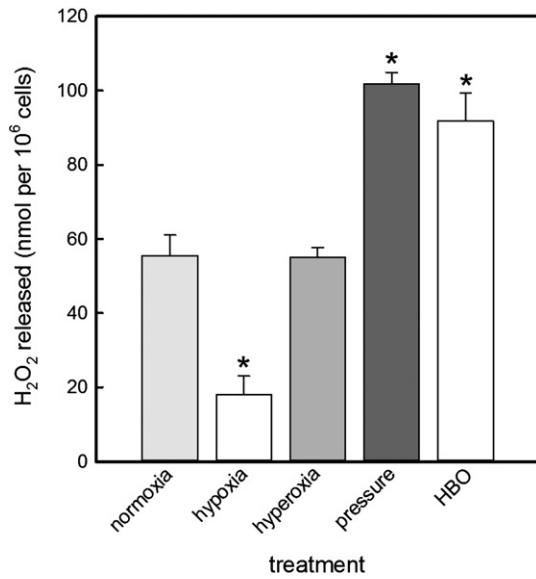


Fig. 2. Effects of oxygen treatments on H₂O₂ production by neutrophil-like cells. After exposure, ATRA-differentiated HL-60 cells were washed, stimulated with PMA and H₂O₂ levels in the medium were determined using HRP/homovanillic acid as described in the **Materials and Methods**. Data are means \pm SEM for three separate experiments (passage numbers 30, 32 and 34) with triplicate measurements ($n = 3$). 1-way ANOVA, $P = 0.0002$. * significant difference versus normoxia (Tukey's HSD, $P < 0.05$).

associated with apoptosis were seen after exposure to HBO, pressure and hyperoxia in the absence of TNF- α stimulation. There was no significant difference in percentage of apoptotic cells after hypoxia pre-treatment compared to normoxia (Fig. 7).

Discussion

The HL-60 cell line, when differentiated using all-*trans* retinoic acid (ATRA) or DMSO, has been used to study neutrophil functions such as oxidative burst, adhesion, chemotaxis and migration (Collins et al., 1979; Martin et al., 1990). Here, ATRA-differentiated HL-60 cells

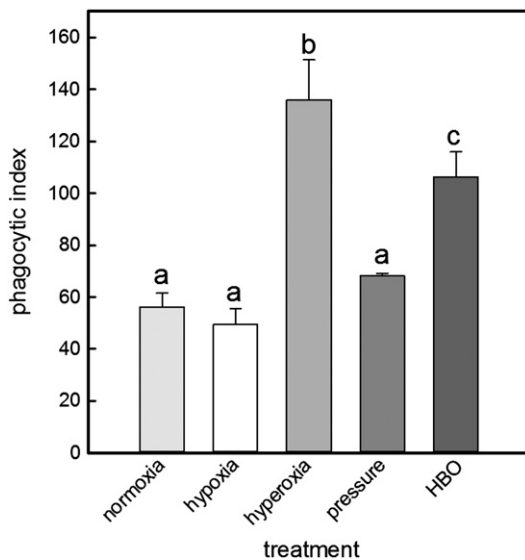


Fig. 3. Effects of oxygen treatments on phagocytosis of *Staphylococcus aureus* by neutrophil-like cells. After exposure, ATRA-differentiated cells were washed and incubated for 30 min with *S. aureus* NCIMB 6571. Phagocytosis (phagocytic index) was assessed as described by Campbell et al. (2001). Data are means \pm SEM for three separate experiments (passage numbers 39, 42 and 45) with triplicate measurements ($n = 3$). 1-way ANOVA, $P = 0.0002$. Bars with different letters are significantly different (Tukey's HSD, $P < 0.05$).

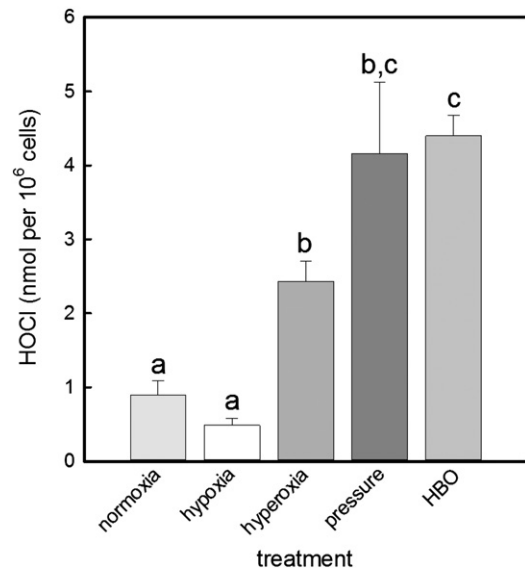


Fig. 4. Effects of oxygen treatments on PMA-stimulated MPO release by neutrophil-like cells. After exposure, ATRA-differentiated cells were washed and PMA-induced MPO activity was measured as described in the **Materials and Methods**. Data are means \pm SEM for three separate experiments (passage numbers 19, 22 and 25) with triplicate measurements ($n = 3$). Kruskal-Wallis, $P = 0.0155$. Bars labelled with different letters are significantly different.

(neutrophil-like cells) were used as a model to study the effects of HBO on antimicrobial activity and apoptosis. Both normobaric hypoxia (5% O₂) and normoxia (21% O₂) were used as controls to encompass the range of oxygen concentrations experienced by circulating neutrophils. Neutrophils in chronic wounds would experience lower levels of oxygen than these, as well as the presence of range of other factors including high levels of lactate and inflammatory mediators (Gill and Bell, 2004).

Effects of hyperbaric oxygen (HBO) on the antimicrobial activity of neutrophil-like cells

Pre-treatment with HBO enhanced the PMA-induced respiratory burst activity of neutrophil-like cells (increased production of O₂⁻ and H₂O₂, Figs. 1 and 2, respectively) compared to normoxia and hypoxia-treated cells. Note, that this was not a direct effect of oxygen on NADPH oxidase activity because when the measurements were made the cells had been returned to normoxia and washed. These results are consistent with those of Labrousche et al. (1999) who found that circulating neutrophils isolated from volunteer divers, who had intermittently inhaled 100% O₂ for 135 min at 1.8 ATA, showed increased bacteria-induced phagocytosis and respiratory burst activity. Similar results were obtained in a study on neutrophils from patients having HBO therapy during treatment for diabetic foot infections (Top et al., 2007). However, other studies are not consistent with these findings. For example, Jüttner et al. (2003) found no effect on respiratory burst or phagocytic activity of neutrophils isolated from healthy volunteers given repetitive HBO exposures. The reason for this discrepancy is unclear, but may in part be down to differences in the methodology used for measuring respiratory burst and phagocytic activity. For example, Jüttner et al. used intracellular oxidation of dihydrorhodamine to detect respiratory burst related ROS via flow cytometry, whereas here, the production of specific ROS (O₂⁻ and H₂O₂) was detected extracellularly.

Hypoxia-inducible factor (HIF) has been implicated in the regulation of the antimicrobial activity of granulocytes, including neutrophils (Peyssonnaud et al., 2005). Regulation of steady state levels of HIF-1 α is oxygen-dependent, but there is a debate over how oxygen affects levels of this protein. Two views, both involving mitochondria,

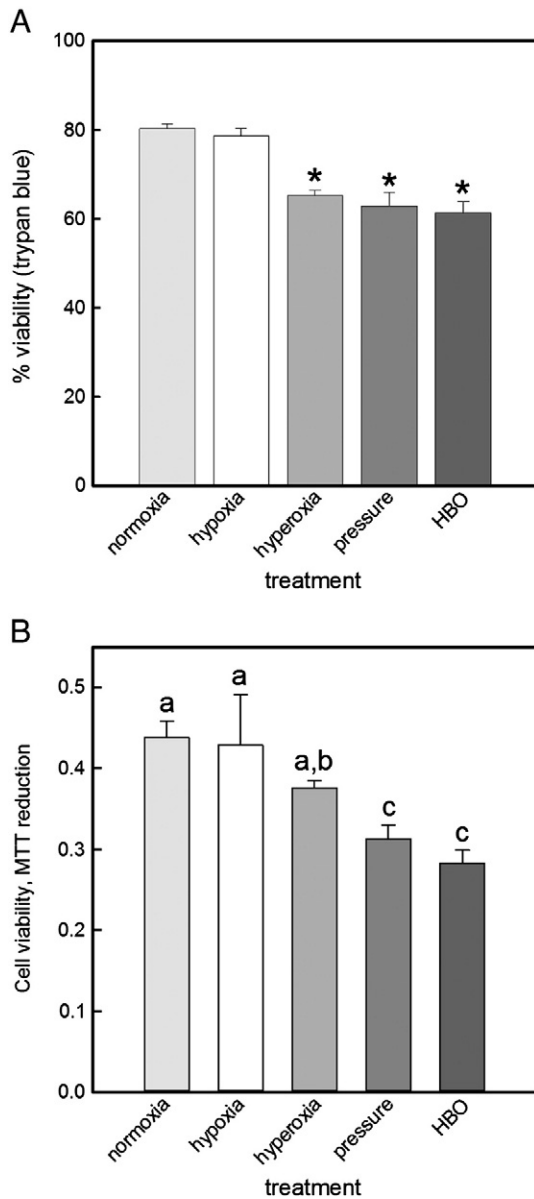


Fig. 5. Effects of oxygen treatments on viability of neutrophil-like cells. ATRA-differentiated cells were exposed to a range of oxygen conditions for 90 min (see [Materials and Methods](#)) after which their viability was assessed using trypan blue (A) or using MTT (B) (see [Materials and Methods](#)). Data are means \pm SEM for three separate experiments (passages 20, 24 and 28) in triplicate ($n = 3$). (A) 1-way ANOVA, $P = 0.0001$. * significant difference versus normoxia (Tukey's HSD, $P < 0.05$). (B) Kruskal–Wallis, $P = 0.0277$. Bars labelled with different letters are significantly different.

have been proposed, one involves modulation of intracellular oxygen concentrations by mitochondria, which in turn affects oxygen-dependent hydroxylation of HIF-1 α (Chua et al., 2010), and the other involves the production of reactive oxygen species (ROS) by mitochondria (Schroedl et al., 2002). HL-60 cells have fully functional mitochondria, but after differentiation to neutrophil-like cells using DMSO they show morphological changes (shift from punctate to tubular structure) and functional decline characteristic of neutrophils (Maianski et al., 2004b). Hence it seems unlikely that mitochondria in neutrophil-like cells would significantly affect intracellular oxygen levels, although they may be potent ROS producers (Fossati et al., 2003). Future investigation into the effects of oxygen on HIF-1 signalling in neutrophil-like cells may resolve this issue, and help elucidate the mechanism of the HBO-induced enhancement of antimicrobial activity seen here.

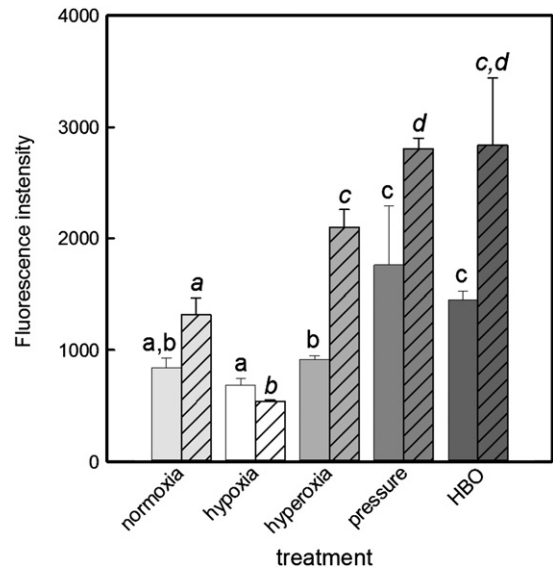


Fig. 6. Effects of oxygen treatments on caspase-3/7 activity in neutrophil-like cells. After exposure, ATRA-differentiated cells were washed and incubated in the absence (bars without hatching) or presence of TNF- α (bars with hatching), for 3 h at 37 °C. The caspase 3/7 activity was then assayed fluorometrically as described in the [Materials and Methods](#). Data are means \pm SEM for three experiments (passages 33, 35 and 37) with duplicate measurements ($n = 3$). For each set of data (i.e. –TNF- α and +TNF- α), bars labelled with different letters are significantly different (Kruskal–Wallis, $P = 0.0209$ and 0.0166 , respectively).

Pre-treatment with HBO enhanced the phagocytic activity of neutrophil-like cells (Fig. 3) and produced an increase in PMA-induced MPO release (Fig. 4), i.e. evidence of degranulation (exocytosis). Phagocytosis requires a controlled rearrangement of the actin cytoskeleton (May and Machesky, 2001); the same is true of exocytosis. For example, Mitchell et al. (2008) found that depolymerisation of cortical F actin is essential for the exocytosis of primary granules, which contain MPO. It has been shown that HBO inhibits adhesion of circulating neutrophils via inhibition of B₂ integrins (Thom, 1993) and evidence has been presented that this is mediated via nitrosylation of actin, which is ultimately dependent on HBO-induced increase in NO production (Thom

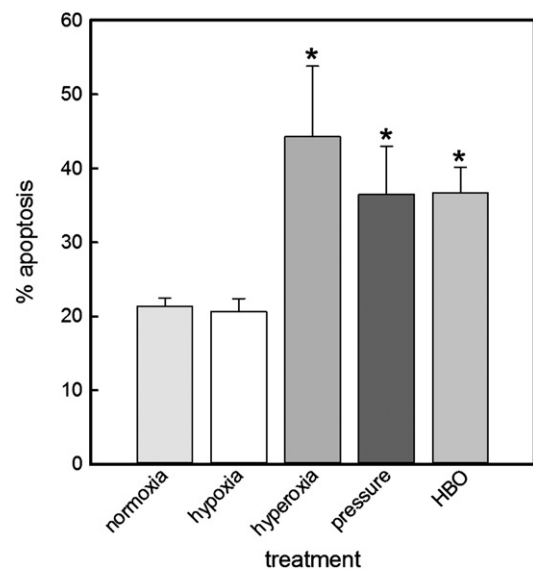


Fig. 7. Effects of oxygen treatments on morphological changes associated with apoptosis in neutrophil-like cells. After exposure, ATRA-differentiated cells were washed, and the percentage of apoptotic cells was assessed morphologically using SYBR Safe staining. Data are means \pm SEM for three separate experiments (passages 27, 29 and 32) in triplicate ($n = 3$). * significant difference versus normoxia (Kruskal–Wallis, $P = 0.0323$).

et al., 2008). The nitrosylation of actin was shown to influence the polymerisation of actin. Hence, this could be the basis of an explanation for the enhancement of phagocytic activity following HBO pre-treatment seen here.

There is also potential for HBO-induced effects to be mediated by pressure (Grim et al., 1990). There was no consistent pattern of effect of pressure on the antimicrobial activity of neutrophil-like cells. On one hand, the increase in H₂O₂ production following HBO pre-treatment could be due to pressure alone, since normobaric hyperoxia had no significant effect (Fig. 2). Similar could be said of the HBO-induced increase in MPO activity; pressure alone had a similar effect to HBO, and although in this case hyperoxia alone had a significant effect it was substantially less than that seen with pressure alone (Fig. 4). On the other hand, it appears that pressure might have an inhibitory effect on phagocytosis since a greater increase in phagocytic activity was seen after treatment with normobaric hyperoxia compared to HBO, and pressure alone had no significant effect (Fig. 3).

Two general mechanisms by which pressure could influence the activity of neutrophils are (a) via deformation during the treatment and (b) via formation of microbubbles during decompression, both of which could activate mechanoreceptors (Makino et al., 2006). There have been some studies, relating to the filtration of neutrophils via capillary beds, in which the effects of their mechanical deformation have been examined. For example, Kitagawa et al. (1997) found that passage of PMN through a 3 µm filter deformed the cells with concomitant increase in F-actin content, together with an increase in cell surface expression of CD18/CD11b (integrin α_Mβ₂ or Mac-1), which has a key role in mediating the adhesion and migration of neutrophils (Solovjov et al., 2005). In contrast, Kitagawa et al. found that filtration had no effect on H₂O₂ production by the cells and that the pressure required to force the cells through the filter had no effect on surface CD18/CD11b levels, although the pressures involved were much lower than that used here.

Neutrophils have also been studied in the context of decompression sickness (DCS). DCS is thought to be caused by the formation of gas bubbles in tissue and the circulation during decompression (Eftedal, 2007). In a pig model of DCS, neutrophil activation and aggregation in tissues were strongly correlated with the neurological symptoms of DCS (Nyquist et al., 2004) and neutrophil activation in DCS may be exacerbated by the formation of microparticles as a result of decompression stress (Thom et al., 2011). Although care was taken in the current work to try to avoid microbubble formation, by decompressing the chambers over 8–10 min, it is still possible that they were a factor in the pressure effects observed.

Effects of HBO on apoptosis of neutrophil-like cells

It seems unlikely that enhancement of the antimicrobial activity of neutrophils would be of benefit for the resolution of inflammation in chronic wounds. However, antimicrobial activity in neutrophils is associated with apoptosis (Watson et al., 1996; Kobayashi et al., 2003; Nakazato et al., 2007) and the benefits of an increase in death of neutrophils by apoptosis are clear, since chronic wounds are characterised by the persistence of an inflammatory and hypoxic environment in which the longevity of neutrophils is increased (Yager and Nwomeh, 1999). In the work described here HBO pre-treatment led to a decrease in viability of neutrophil-like cells (Fig. 5), and an increase in apoptosis as assessed using caspase 3/7 activity and fluorescence staining using SYBR Safe (Figs. 6 and 7, respectively). These results are consistent with the effects of HBO previously demonstrated in a range of haematopoietic cells including undifferentiated HL-60 cells, Jurkat cells and murine thymocytes (Ganguly et al., 2002). For example, in HL-60 cells, HBO increased both spontaneous and γ irradiation-induced apoptosis, but normobaric hyperoxia or pressure alone had little effect.

There is evidence that the increase in apoptosis associated with antimicrobial activity in neutrophils is a consequence of increased ROS production by the cells themselves (Watson et al., 1996; Nakazato et al., 2007). In addition, as discussed above, mitochondria are likely to have a role both in ROS production and in the intrinsic apoptotic pathway (Maianski et al., 2004a). Presumably in our experiments there was an elevated mitochondrial ROS production during the HBO treatment in addition to the potential for elevated antimicrobial ROS production post HBO treatment (Fig. 1A, B and D).

Conclusion

In conclusion, exposure of HL-60 cells differentiated with ATRA (neutrophil-like cells) to hyperbaric oxygen (HBO) enhanced the antimicrobial activity of the cells as well as promoting apoptosis. The former could be regarded as detrimental to wound healing in chronic wounds because it promotes a pro-inflammatory environment. However, this may be offset by the latter, since neutrophil apoptosis is of key importance to the resolution of inflammation during wound healing. Both the hyperoxia and pressure components of HBO contribute to effects on neutrophil-like cells.

Conflict of interest statement

Gary Smerdon is the CEO and Research Director of DDRC, a charity dedicated to research, education and treatments involving altered pressure and gas environments.

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