Hyperbaric Oxygen Inhibits Stimulus-Induced Pro-Inflammatory Cytokine Synthesis by Human Blood-Derived Monocyte-Macrophages

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Hyperbaric oxygen inhibits stimulus-induced proinflammatory cytokine synthesis by human blood-derived monocyte-macrophages

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SUMMARY

Hyperbaric oxygen (HBO) is 100% oxygen administered at elevated atmospheric pressure to patients with inflammatory diseases. We developed an in vitro model to investigate the effects of HBO on stimulus-induced proinflammatory cytokine transcription and translation. Human blood-derived monocyte-macrophages were stimulated before being transferred to an HBO chamber where they were incubated at 97.9% O₂, 2.1% CO₂, 2.4 atmospheres absolute, 37°C. Controls were maintained in the same warm room at normoxia or increased pressure alone. A 90-min HBO exposure inhibited IL-1β synthesized in response to lipopolysaccharide by 23%, lipid A by 45%, phytohaemagglutinin A (PHA) by 68%, and tumour necrosis factor (TNF-α) by 27%. HBO suppressed lipopolysaccharide-, lipid A- and PHA-induced TNF-α by 29%, 31% and 62%, respectively. HBO transiently reduced PHA-induced steady state IL-1β mRNA levels. Hyperoxia alone and pressure alone did not affect cytokine production. The immunosuppressive effect of HBO was no longer evident in monocyte-macrophages exposed to HBO for more than 3 h. Interestingly, cells exposed to HBO for 12 h synthesized more IL-1β than cells cultured under control conditions. In summary, HBO exposure transiently suppresses stimulus-induced proinflammatory cytokine production and steady state RNA levels.

Keywords cytokine hyperbaric oxygenation interleukin 1 monocyte tumour necrosis factor

INTRODUCTION

Interleukin (IL)-1 and tumour necrosis factor (TNF-α) are present in the tissues or the systemic circulation in many inflammatory conditions. While these proinflammatory cytokines contribute to the elimination of invading pathogens, inappropriate production of these molecules can be deleterious. IL-1 and TNF have been shown to mediate inflammation in animal models of inflammatory arthritis [1,2]. Inhibiting IL-1 and TNF-α ameliorates joint disease in humans with rheumatoid arthritis [3,4]. Non-specific, endogenous inhibitors of these molecules include glucocorticoids and anti-inflammatory cytokines such as IL-10. Specific inhibitors exist in the form of receptor antagonists and soluble cytokine receptors.

Hyperbaric oxygen (HBO) therapy provides patients with 100% inhaled oxygen at increased atmospheric pressure. HBO is used as adjuvant therapy for various inflammatory conditions, including necrotizing soft tissue infection, gas gangrene, refractory osteomyelitis, burns and chronic wounds [5]. Although HBO has been shown to enhance some aspects of host defence, its overall effect appears to be immunosuppressive [6]. More specifically, HBO impairs macrophage function [7].

The monocyte-macrophage is an important source of IL-1 and TNF-α. Recently, it was shown that HBO enhances cytokine release from lipopolysaccharide (LPS)-stimulated murine macrophages [8]. Monocyte-macrophages isolated from HBO-exposed rodents produce less proinflammatory cytokines than cells from unexposed controls [9,10]. Monocyte-macrophages from patients with Crohn’s disease secrete less IL-1, IL-6 and TNF-α in response to LPS when isolated after HBO therapy than cells obtained prior to the treatment [11]. None of these ex vivo investigations controlled for the isolated effects of hyperoxia or increased atmospheric pressure, used stimuli other than LPS, studied the effect of duration of exposure or examined the effects of HBO on transcription.

In this study, we placed both a small animal HBO chamber and a second sealed chamber in a 37°C warm room. By changing the gas mixtures supplying the chambers, we were able to control for the effects of oxygen alone. We used this model to investigate...
the effects of HBO on stimulus-induced proinflammatory cytokine synthesis and steady state RNA levels in human blood-derived monocyte-macrophages.

MATERIALS AND METHODS

Reagents
LPS (from Escherichia coli O55:BS) and lipid A (from Salmonella minnesota Re 595) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Phytohaemagglutinin A (PHA) was obtained from Difco Laboratories (Detroit, MI, USA). Human recombinant TNF-α was generously supplied by Genentech Inc. (South San Francisco, CA, USA). RPMI-1640 (GIBCO BRL, Gaithersburg, MD, USA) with 10 mM glutamine, 24 mM NaHCO₃ and 25 mM HEPES contained <3 EU/ml. RPMI was supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO). Human AB serum collected aseptically from one donor was inactivated AB serum. PBMC (2·5 × 10⁶ cells/ml) were incubated at 37∞C, 21% O₂, 5% CO₂ and pressurized to 2·4 atmospheres absolute (ATA) so that the O₂ concentration would be the equivalent of 21% O₂ at 1 ATA. We used 2·1% CO₂ and pressurized to 2·4 atmospheres absolute (ATA) (equivalent to 46 feet sea water) over 5 min; 2·4 ATA was chosen because HBO treatments are commonly administered at this pressure. We used 2·1% CO₂ because cells incubated at 2·4 ATA in an atmosphere containing this concentration of CO₂ are exposed to the equivalent of 5% CO₂ at 1 ATA (sea level). To ascertain the effect of pressure alone, cells were incubated at 37∞C in 8·75% O₂, 2·1% CO₂ and pressurized to 2·4 atmospheres absolute (ATA) (equivalent to 46 feet sea water) over 5 min; 2·4 ATA was chosen because HBO treatments are commonly administered at this pressure. We used 2·1% CO₂ because cells incubated at 2·4 ATA in an atmosphere containing this concentration of CO₂ are exposed to the equivalent of 5% CO₂ at 1 ATA (sea level). To ascertain the effect of pressure alone, cells were incubated at 37∞C in 8·75% O₂, 2·1% CO₂ at 2·4 ATA. We used 8·75% O₂ so that the O₂ concentration would be the equivalent of 21% O₂ at 1 ATA. To determine the effect of hyperoxia alone, cells were incubated at 37∞C in a chamber containing 95% O₂, 5% CO₂ at 1 ATA. ‘Control’ cultures for each experiment were placed in an incubator at 37∞C, 21% O₂, 5% CO₂ at 1 ATA. Following exposure to HBO, increased atmospheric pressure or hyperoxia, plates were removed from the chambers and transferred to the 37∞C, 21% O₂, 5% CO₂, 1 ATA incubator to complete a 24-h incubation. The chambers as well as the incubator were humidified. After incubation, cultures were frozen immediately at −70∞C (unless separated).

Where indicated, monocyte-macrophages and supernatants were separated before freezing. Supernatants were transferred to 1·5 ml polypropylene microtube tubes and centrifuged at 300 g for 5 min. The resulting supernatants were then transferred to new microtube tubes and frozen at −70∞C. New RPMI with 1% AB serum was added to each well and the plates were also frozen at −70∞C.

Plates were subjected to three freeze-thaw cycles to yield maximal recovery of IL-1β and TNF-α by enzyme immunoassays (R&D Systems, Minneapolis, MN, USA). The limits of detection for the enzyme immunoassays were 4 pg/ml IL-1β and 16 pg/ml TNF-α.

RNA isolation
PBMC (1 × 10⁷ cells in 2 ml RPMI with 2% AB serum) were aliquoted into either a 50-ml polypropylene tube or the wells of 6-well polystyrene plates. Two millilitres of either RPMI or PHA in RPMI (10 µg/ml final concentration) was added. Some plates were incubated in the HBO chamber at 97·9% O₂, 2·1% CO₂, 2·4 ATA; the remaining samples were cultured in an incubator at 95% air, 5% CO₂, 1 ATA. After 1·5 or 6 h of incubation, tissue culture plates were placed on wet ice. RNA was isolated individually from adherent and non-adherent cells in each well, then pooled for analysis. For non-adherent cells, medium containing dislodged cells was collected from each well, transferred to clean polypropylene microtube tubes and spun at 200 g for 5 min at 4∞C to pellet cells. Supernatants were removed and cells were resuspended in 1 ml of cold RNAzol B (TelTest, Friendswood, TX, USA) with vigorous pipetting. Cells were lysed in RNAzol B for 5 min on wet ice. For adherent cells, 2 ml of cold RNAzol B was added to each culture well, and cells were lysed for 2 min in the wells. Plates were tipped slightly and well bottoms were rinsed gently by pipetting to fully dislodge/lyse adherent cells. Lysates were transferred in 1-ml aliquots to clean polypropylene microfuge tubes, and incubated on wet ice for 5 min. For both adherent and non-adherent cells, 200 µl of chloroform was added to each 1-ml aliquot of cell lysate and the mixture incubated for an additional 5 min on wet ice. Samples were spun at 16 000 g for 15 min at 4∞C and the aqueous phase was removed and pooled for each test condition. A second chloroform extraction was performed, and the aqueous phase recovered and divided into 600 µl aliquots. Total RNA was precipitated overnight at −70∞C by the addition of 0·5 volume of ice-cold isopropanol to each aliquot. Precipitated RNA was centrifuged at 16 000 g for 15 min at 4∞C to pellet. Supernatants were removed and the pellets washed twice in 75% (v/v) ethanol in diethylpyrocarbonate-treated H₂O. Pellets were dried under gentle vacuum for 5 min before being resuspended in 20 µl of diethylpyrocarbonate-treated H₂O. RNA concentrations were determined spectrophotometrically by measuring the optical density of diluted samples at 260 nm. Serial dilutions of RNA were applied to wells of a multichamber manifold, and transferred onto a nylon membrane (Magna Charge Nylon, Micron Separations, Inc., Minnetonka, MN, USA) using the methods of White and Bancroft [14]. Prior to hybridization, RNA was cross-linked to membranes using short-wave ultraviolet radiation.

Hybridization
The IL-1β probe was a 1·047-kb fragment of the full-length complementary DNA for the human IL-1β precursor (American Type
Culture Collection, Manassas, VA, USA). The β-actin probe was a 1.4-kb fragment of the full-length complementary DNA for human β-actin (American Type Culture Collection). DNA was labelled with 32P-CTP by using an oligolabelling kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Prehybridization, hybridization, washing and autoradiography were performed using standard techniques [15]. Prior to using the IL-1β probe for experiments, its specificity and sensitivity were verified by Northern blot and then dot-blot analysis using serial dilutions of total RNA isolated from PHA-stimulated PBMC, unstimulated PBMC and a negative control mammary epithelial EPH4 line which does not transcribe IL-1β. In stimulated cells, IL-1β mRNA expression was detected at a concentration of total RNA as low as 0.28 μg.

Statistics
Statistical analysis was performed using the Student’s t-test for paired samples and analysis of variance using Fisher’s least significant difference. Data are expressed as mean ± standard error of the mean (s.e.).

RESULTS

HBO inhibits stimulus-induced IL-1β and TNF-α production in human blood-derived monocyte-macrophages
In the present study we investigated whether HBO could interfere with stimulus-induced cytokine synthesis. Human blood-derived monocyte-macrophages were stimulated with LPS, lipid A, PHA or TNF-α and then incubated for 90 min in either 95% air, 5% CO2 at sea level (normoxia at sea level) or 97.9% O2, 2.1% CO2 at 2.4 ATA (HBO). As shown in Table 1, HBO inhibited IL-1β synthesized in response to LPS by 23% (P = 0.006), lipid A by 45% (P < 0.001), PHA by 68% (P = 0.001) and TNF-α by 27% (P < 0.001). HBO suppressed LPS-, lipid A- and PHA-induced TNF-α by 29% (P < 0.001), 31% (P = 0.001) and 62% (P = 0.001), respectively.

When PBMC are cultured at sea level, stimulus-induced IL-1β and TNF-α are predominantly secreted [12]. In this study, exposing PHA-stimulated cells to HBO neither enhanced nor inhibited the secretion of IL-1β or TNF-α. HBO suppressed cytokine levels in both the extracellular and intracellular compartments (data not shown).

Hyperoxia alone does not affect stimulus-induced cytokine synthesis
To determine whether hyperoxia alone can inhibit cytokine production, an apparatus was constructed whereby PBMC could be cultured concurrently in 95% O2, 5% CO2 at sea level (hyperoxia) or HBO. Because the preceding data showed that HBO had its most profound effect upon PHA-induced cytokines, PHA was chosen as the stimulus for the subsequent experiments. When compared to PHA-stimulated monocyte-macrophages cultured in normoxia at sea level, HBO-exposed monocyte-macrophages again synthesized 57% less IL-1β and 60% less TNF-α (both P < 0.001). Table 2 shows that 90 min of hyperoxia did not affect PHA-induced IL-1β or TNF-α. A 12-h exposure to hyperoxia also had no effect upon cytokine production (data not shown).

Increased atmospheric pressure alone does not affect stimulus-induced cytokine synthesis
Previous investigations have shown that increased atmospheric pressure can affect such cellular functions as interferon-γ secretion [16] and apoptosis [17,18]. To assess the effects of increased atmospheric pressure on cytokine production, monocyte-macrophages were cultured in 8.75% O2, 2.1% CO2 at 2.4 ATA (increased atmospheric pressure). We used 8.75% O2 and 2.1% CO2 so that cells at 2.4 ATA would be exposed to the equivalent of 21% O2, 5% CO2 at sea level. When compared to cells cultured in normoxia at sea level, up to 12 h of increased atmospheric pressure did not affect IL-1β or TNF-α synthesis (data not shown).

The effect of HBO on stimulus-induced cytokine synthesis depends upon the duration of exposure
The preceding experiments showed that IL-1β and TNF-α synthesis is inhibited by a 90-min HBO exposure. To determine the effects of the duration of HBO exposure, PHA-stimulated monocyte-macrophages were incubated at 2.4 ATA for up to 12 h. As shown in Fig. 1a, a 30-min HBO exposure inhibited IL-1β synthesis by 73% (P = 0.001). Interestingly, if the cells remained in the

Table 1. HBO inhibits stimulus-induced IL-1β and TNFα production in human monocyte-macrophages

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>IL-1β</th>
<th>TNF-α</th>
</tr>
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<tbody>
<tr>
<td>LPS</td>
<td>77 ± 8*</td>
<td>71 ± 7**</td>
</tr>
<tr>
<td>Lipid A</td>
<td>55 ± 6**</td>
<td>69 ± 8**</td>
</tr>
<tr>
<td>PHA</td>
<td>32 ± 12**</td>
<td>38 ± 9**</td>
</tr>
<tr>
<td>TNF-α</td>
<td>73 ± 3**</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Human blood-derived monocyte-macrophages were cultured with LPS (100 ng/ml), lipid A (100 ng/ml), PHA (10 μg/ml), or TNF-α (100 ng/ml). Immediately after stimulation, cells were incubated for 90 min at 37°C in either 95% air, 5% CO2 at 1 ATA (control) or 97.9% O2, 2.1% CO2 at 2.4 ATA (HBO). All samples were then incubated for another 22.5 h at 37°C in 95% air, 5% CO2 at 1 ATA. Enzyme immunosassays were performed to determine the total concentration of IL-1β and TNF-α. Concentrations of cytokine synthesized by cells exposed to HBO are expressed as a percentage of cytokine concentration produced by control cells. IL-1β and TNF-α are depicted as the mean ± s.e. of four and six donors, respectively. *P = 0.006, **P ≤ 0.001 and ***P < 0.001 when comparing cells exposed to HBO with unexposed cells using the paired t-test. n.d., not done.

Table 2. Hyperoxia alone does not affect stimulus-induced cytokine synthesis

<table>
<thead>
<tr>
<th>Condition</th>
<th>IL-1β</th>
<th>TNF-α</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Hyperoxia</td>
<td>95 ± 5</td>
<td>107 ± 8</td>
</tr>
<tr>
<td>HBO</td>
<td>43 ± 2***</td>
<td>40 ± 10**</td>
</tr>
</tbody>
</table>

Monocyte-macrophages were stimulated with PHA (10 μg/ml) before being incubated for 90 min at 37°C in either 95% air, 5% CO2 at 1 ATA (control), 95% O2, 5% CO2 at 1 ATA (hyperoxia) or 97.9% O2, 2.1% CO2 at 2.4 ATA (HBO). All samples were then incubated for another 22.5 h at 37°C in 95% air, 5% CO2 at 1 ATA. Concentrations of cytokine synthesized by cells exposed to hyperoxia or HBO are expressed as a percentage of cytokine concentration produced by control cells. IL-1β and TNF-α are depicted as the mean ± s.e. of four donors. **P = 0.001 and ***P < 0.001 when comparing cells exposed to HBO with control using the paired t-test.
In PHA-stimulated cells, there was a marked increase in IL-1 expression associated with PHA (10 µg/ml) compared to a non-stimulus-induced cytokine synthesis. Monocyte-macrophages were stimulated with PHA (10 µg/ml) in either a 95% air, 5% CO2, 1 ATA incubator or a 97.9% O2, 2.1% CO2, 2.4 ATA HBO chamber (lanes 2–9). Control PBMC were not exposed to polystyrene to demonstrate that the isolation procedure did not induce IL-1β gene expression (lane 1). Total RNA was extracted at the specified times, applied to a nylon membrane, and hybridized to the IL-1β or β-actin probe. Similar results were obtained using a second blood donor.

HBO inhibits PHA-induced early IL-1β gene expression

To determine whether HBO inhibits PHA-induced IL-1β production by affecting transcription, PBMC were incubated with PHA and then exposed to HBO for 1.5 or 6 h. As shown in Fig. 2, maintaining PBMC in a polypropylene tube did not induce IL-1β gene expression (lane 1), but culturing cells in a polystyrene plate resulted in a transient increase in steady state IL-1β mRNA (lane 2). In PHA-stimulated cells, there was a marked increase in IL-1β mRNA (lanes 4 and 8). HBO exposure reduced PHA-induced IL-1β mRNA (lane 4 compared to lane 5). In cells cultured for 6 h, the suppressive effect of HBO was no longer evident (lane 8 compared to lane 9). Similar results were obtained using a second donor (data not shown).

**DISCUSSION**

A striking conundrum is evident immediately when one reviews studies on the effects of oxygen on cytokine synthesis. Most investigators have found that hyperoxia enhances proinflammatory cytokine transcription and translation [19–23]. In contrast, HBO has generally been shown to suppress stimulus-induced proinflammatory cytokine production [6]. The experiments presented here provide some insight into this apparent contradiction.

In this study, we placed both a small animal HBO chamber and a second sealed chamber in a 37°C warm room. By changing the gas mixtures supplying the chambers, we were able to control for the effects of oxygen alone. While previous investigators used animal HBO chambers to study the in vitro effects of HBO on cell proliferation [24–26] or cytokine release [8], they performed their experiments at room temperature, excluded CO2 from the HBO gas mixture, or did not control for the effects of hyperoxia alone.

No previous in vitro or ex vivo study has examined the effects of HBO on cytokine gene expression. We used our model to examine the effects of HBO on monocyte-macrophage proinflammatory cytokine transcription and translation. We found that HBO suppressed LPS-, lipid A- and PHA-induced IL-1β and TNF-α production. Suppression of PHA-induced cytokine synthesis and mRNA levels was evident during the first 90 min of the exposure. Thereafter, the inhibitory effect of HBO began to wane. Transient exposure to 95% O2 at standard atmospheric pressure or normoxia at increased atmospheric pressure had no effect upon monocyte-macrophage proinflammatory cytokine production.

The results of our in vitro experiments are consistent with previous ex vivo studies demonstrating that monocyte-macrophages isolated from HBO-exposed rodents produce less proinflammatory cytokines than cells from controls exposed to air at standard atmospheric pressure. Inamoto and colleagues [9] exposed mice to 2.5 ATA for 60 min daily for 5 days before sacrificing the animals and culturing their splenic macrophages in the presence of LPS. IL-1 bioactivity was markedly decreased in cultures from HBO-exposed mice as compared to unexposed controls. After exposing rats to 100% O2 for 90 min at 2.8 ATA, Lahat et al. [10]
isolated peripheral blood monocyte-macrophages and then cultured the cells with LPS. Monocyte-macrophages from HBO-exposed rats secreted less TNF-α than cells from rats exposed to air. In patients with Crohn’s disease treated with 100% O₂ for 90 min at 2.5 ATA, LPS-stimulated monocyte-macrophages isolated following the exposure secreted less IL-1, IL-6 and TNF-α than cells obtained prior to the treatment [11]. Comparing whole blood cultures obtained from normal, healthy volunteers before and after a 90-min exposure to 100% O₂ at 2 ATA, we observed a decrease in ex vivo stimulus-induced interferon-γ secretion [16].

Our findings are also in agreement with studies demonstrating that HBO attenuates proinflammatory cytokine production in vivo in animal models of systemic inflammation. In a murine zymosan-induced shock model, plasma concentrations of TNFα were significantly reduced in animals exposed to 100% O₂ for 60 min at 2 ATA compared to unexposed controls [27]. Following massive haemorrhage, hepatic TNF-α mRNA and circulating TNF-α levels were lower in rats exposed for 60 min to 3 ATA than in unexposed controls [28]. Exposing rats to 100% O₂ for 60 min at 2.8 ATA also inhibited intestinal ischaemia–reperfusion-induced circulating TNF-α [29].

These previous ex vivo and in vivo models all reported the results of either a single or repeated daily HBO exposures each lasting 90 min or less. Interestingly, when we extended a single HBO exposure beyond 3 h, its cytokine-suppressive effect waned and after 12 h there was an augmentation of stimulus-induced cytokine production. This observation is reminiscent of studies examining the effects of normobaric hyperoxia on cytokine synthesis. After exposing mice to hyperoxia, several groups have detected significant increases in IL-1 [19,22,23] and TNF-α [19–22] gene expression in the pulmonary parenchyma and cells recovered from bronchoalveolar lavage. However, these effects were not evident until the animals had received at least 48 h of hyperoxia. This suggests that oxygen-induced inflammation is a function of both the partial pressure of oxygen and the duration of exposure. Once a certain threshold is reached, antioxidant defences may become overwhelmed and inflammation ensues. In blood-derived monocyte-macrophages, we found that 12 h of HBO, but not 95% O₂, enhanced PHA-induced proinflammatory cytokine synthesis. In light of the rodent hyperoxia data, we suspect that the intensity of hyperoxia administered to the HBO-exposed monocyte-macrophage was the cause of the enhanced cytokine synthesis. How does HBO inhibit stimulus-induced proinflammatory cytokine production? The inhibitory effect of HBO seen in the ex vivo and in vivo models could be due to changes in the distribution of mononuclear cell subsets [30,31]. However, this is not the case in our model which used a fixed cell population. In some models, HBO could reduce cytokine production by inducing apoptosis in cytokine-producing cells. For example, HBO stimulates apoptosis in murine thymocytes as well as lymphocytic and granulocytic cell lines [18]. However, in this study the inhibitory effects of HBO on cytokine synthesis were only transient, thereby suggesting that HBO-induced a qualitative change in the monocyte-macrophage. This change is probably mediated by interference or augmentation of an early signalling event. For example, HBO could inhibit cytokine production by down-regulating PGE₂ production [9] via suppression of Cox-2 expression [32] or by inducing the formation of heat shock proteins [33].

Our findings raise a very important clinical question. What is the optimal duration of any one HBO treatment? The data presented here suggest that relatively short HBO treatments may be more immunosuppressive than more prolonged exposures. However, conclusions based on our results have limitations. Obviously, in vitro observations are not always predictive of what occurs in vivo. Although patients often receive 20 or more HBO treatments, we studied the effects of only a single HBO exposure. Due to the availability of only a single HBO chamber, we were unable to perform a dose–response experiment to determine the atmospheric pressure which maximally suppresses cytokine production. Finally, unlike resting monocytes and tissue macrophages which do not transcribe IL-1β or TNF in vivo, our monocyte-macrophages transiently expressed this gene. Although we knew a priori that polystyrene stimulates IL-1β expression [33,34], we cultured monocytes on this surface to induce them to differentiate into macrophages. We chose an in vitro macrophage model, because macrophages have an important role in many diseases treated with HBO.

In summary, we found that HBO exposure transiently suppresses stimulus-induced proinflammatory cytokine production and steady state mRNA levels. Future investigations using our model may allow better elucidation of the mechanisms whereby HBO affects cytokine gene expression in particular and cellular function in general.

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