Whole blood pro-inflammatory cytokines and adhesion molecules post-lipopolysaccharides exposure in hyperbaric conditions

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ABSTRACT. Hyperbaric oxygen (HBO) is a therapeutic intervention with applications in a large variety of diseases, including traumatic injuries and acute or chronic infections. The presence of pro-inflammatory cytokines regulates certain factors including adhesion molecules, which play a significant role in HBO effects.
We have investigated the effect of HBO on pro-inflammatory cytokine release [tumor necrosis factor-α (TNF-α), interleukin 6 and 8 (IL-6 and IL-8)], and the regulation of adhesion molecules [soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular adhesion molecule (sVCAM)] after lipopolysaccharide (LPS) stimulation in 16 healthy individuals, originating from an urban area. A total number of 64 samples were treated, divided into four groups: Group A: not stimulated with LPS and not exposed to HBO. Group B: stimulated with LPS and not exposed to HBO. Group C: not stimulated with LPS and exposed to HBO. Group D: stimulated with LPS and exposed to HBO. The LPS stimulation dose was 100 pg/ml for 0.1ml whole blood diluted 1:10. After incubation, samples were exposed to HBO with 100% O_2 at 2.4 atmospheres absolute (ATA) for 90 min. TNF-α, IL-6, IL-8 and sICAM-1, sVCAM levels were determined in culture supernatant, with ELISA. We observed an enhanced effect of LPS stimulation following exposure to HBO, which caused an increase in cytokine production (TNF-α, IL-6, IL-8), a reduction in sICAM, and no change to sVCAM, while their levels without stimulation remained almost invariable. The decrease in sICAM levels could be related to the increased levels of IL-8, as the production of this chemokine is involved in the regulation of adhesion molecules.

Keywords: cytokine, adhesion molecules, LPS, HBO, whole blood

INTRODUCTION

Hyperbaric oxygen (HBO), enhanced delivery of oxygen to tissues by breathing 100% oxygen in an ambient pressure environment, greater than one atmosphere absolute (ATA), induces compression of any developed bubbles, a pharmaceutical effect on some bacteria, stimulation of immune responses, reversal of carbon monoxide (CO) poisoning and tissue hypoxia, and accelerated healing of injuries and wounds. The increased partial pressure of oxygen (PO_2) influences all tissues by several different mechanisms, including modification of the inflammatory response [1, 2].

Inflammation is known to cause organ dysfunction and cell damage. It is also well known that in normal individuals, whole blood stimulation with lipopolysaccharide (LPS) enhances inflammatory cytokine release, such as tumor necrosis factor-α (TNF-α), interleukins 1, 6 and 8 (IL-1, IL-6 and IL-8)], and affects the levels of soluble adhesion molecules (intercellular adhesion molecule, sICAM-1 and vascular adhesion molecule, sVCAM). ICAM-1 is a well-known marker of acute and chronic inflammation. ICAM-1 serves as the receptor for leukocyte function-associated antigen-1 (LFA-1; CD11a/CD18), a β_2-integrin, which is expressed on neutrophils, monocytes, lymphocytes, and natural killer cells. VCAM interacts with the β_1-integrin very late antigen-4 (CD49d/CD29), which is expressed on lymphocytes and monocytes, but not on neutrophils [3]. IL-8, a chemotactic protein, is involved in the regulation of adhesion molecules, ICAM-1, VCAM, PECAM and selectins. Release of such inflammatory cytokines locally, into the tissue milieu, enhances the tissue's antimicrobial ability and helps its repair, but in certain conditions their overproduction may cause tissue injury [4].

Exposure to HBO can induce immediate alterations in the immune function under normal and pathological conditions. Changes involving cellular and humoral components of the immune system indicate a down-regulation of the immune response, which is generally short-lived. [2].

HBO therapy for five days significantly decreased IL-1 release by murine splenic macrophages, while IL-6 concentrations showed no significant changes [5]. Monocyte-macrophages isolated from HBO-exposed rats released less TNF-α compared to cells isolated from rats not previously exposed to HBO [6]. HBO also inhibits stimulus-induced IL-1β and TNF-α release from human blood-derived monocyte-macrophages [7].

Conversely, Van den Blink *et al.* [8] observed that HBO exposure enhances cytokine release by both non-stimulated as well as LPS-challenged macrophages *in vitro*. TNF-α levels increased significantly during and after a single HBO session in healthy volunteers, while IL-6 and IL-1β levels did not change significantly [9].

We have investigated the effect of HBO on early pro-inflammatory cytokine release (TNF-α, IL-6 and IL-8) and soluble adhesion molecule (sICAM-1 and sVCAM) expression in whole blood samples of healthy individuals after LPS stimulation.

PATIENTS AND METHOD

Blood collection

We included 16 healthy volunteers aged 37.4 ± 1.9 yrs old (range 25-50), all of them residents of an urban area. Six were current light smokers (< 10 cigarettes per day). These individuals were not suffering from any inflammatory condition, did not undertake intensive exercise, did not suffer any illnesses or consume any medications during the study. Women did not take any oral contraceptive or hormone replacement therapy. All of them had normal sleep/wakefulness pattern and their duration of sleep was similar.

All sixteen healthy volunteers served as blood donors (10 ml). Blood samples were collected under sterile conditions via a peripheral vein and at the same time (9.00 am), after the subjects had acclimatized for 60 min at room temperature. Samples were placed in vacutainer tubes containing EDTA as anticoagulant, and were immediately transferred to the laboratory for processing.

Reagents

LPS derived from *E. coli* (0111:B4), Sigma Chemical Co. (St Louis, MO, USA).

Sample groups studied

A total number of 64 samples was used, divided into four groups (16 samples in each Group, one sample from each subject):

Group A: samples not stimulated with LPS, not exposed to HBO (normobaric conditions)

Group B: samples stimulated with LPS, not exposed to HBO (normobaric conditions) Group C: samples not stimulated with LPS and exposed to HBO and

Group D: samples stimulated with LPS and exposed to HBO.

The LPS dose used for stimulation was 100 pg/ml. Samples exposed to HBO were incubated for 2 h under normobaric conditions and then exposed to 100% O_2 in a monoplace hyperbaric chamber (BLKS 303, Krunichef State Center, Moscow, Russia) at 2.4 ATA for 90 min (one session). The pressure of 2.4 ATA was chosen because HBO treatments are commonly administered at this pressure.

Whole blood assay

Cytokine and adhesion molecule induction of whole blood by LPS was evaluated as previously described by others and us [10,11]. Briefly, heparinized blood from healthy volunteer donors was diluted 1:10 in RPMI 1640 culture medium supplemented with 1% L-glutamine 200 mM, 1% FBS (Biochrom) in duplicate. One hundred μL samples of blood were added in 900 μL of RPMI 1640 for a final volume of 1 ml and put into plastic culture dishes.

Whole blood samples from healthy volunteers ($N = 16$) diluted at 1:10, were stimulated with LPS 100 pg/ml, at 37 °C, for 2 h under normobaric conditions and then, exposed to HBO 100% O_2 at 2.4 ATA for 90 min (group D) or to normobaric conditions (group B).

Another batch of samples was not stimulated by LPS but treated as follows: two hours exposure at 37 °C to normobaric conditions and then to HBO 100% O_2 at 2.4 ATA for 90 min (group C) or to normobaric conditions (group A).

Samples were added to wells and maintained at 37 °C in a 5% CO_2 atmosphere. After an incubation period of 120 min, the culture plate was centrifuged (1800 rpm, 10 min) and supernatants were collected and stored at – 80 °C until measurements. Subsequently, cytokine levels in the supernatant were measured using the ELISA method.

Cytokine assays

Levels of soluble TNF-α, IL-6, IL-8, ICAM-1 and VCAM were measured using commercially available human specific enzyme-linked immunoassays kits (DIACLONE, France). All samples were tested in duplicate, according to the instructions, and standard recombinant proteins used as internal controls in each assay. All kits for cytokines were human specific with a sensitivity for detecting levels at < 10 pg/mL, < 2 pg/mL, < 25 pg/mL, < 0.1 ng/mL and < 0.6 ng/mL for TNF-α, IL-6, IL-8, ICAM-1 and VCAM respectively. In addition, serum cytokine levels were measured in each volunteer.

The purpose of the study was to evaluate the effect of LPS stimulation of whole blood taken from healthy volunteers on cytokine release under hyperbaric and normobaric conditions. Specifically, we aimed to evaluate the capacity of whole blood immune cells to respond to endotoxin exposure. This might provide additional information on the immune cell's capacity to produce pro-inflammatory cytokines and adhesion molecules under different oxygen partial pressures.

Statistical analysis

Results are expressed as means ± SEM and compared using one-way analysis of variance (ANOVA) adjusted for multiple comparisons by the Tukey test. A P value < 0.05 was considered to be statistically significant.

RESULTS

Under normobaric conditions, there was a statistically significant increase ($P < 0.001$) concerning pro-

Table 1
HBO increases TNF-α, IL-6 and IL-8 levels in whole blood (0.1 ml) from healthy individuals after LPS stimulation

Cytokines (pg/mL)	Normobaric conditions		Hyperbaric conditions	
	Group A	Group B	Group C	Group D
TNF-α	0.4 ± 0.2	200.9 ± 36*	0.01	360 ± 60.6*
IL-6	0.3 ± 0.2	325.2 ± 83.7**	0.8 ± 0.23	980.1 ± 134.7**
IL-8	2.0 ± 0.6	263.8 ± 65.1***	7.2 ± 2.44	586.2 ± 112.2***

TNF-α, IL-6 and IL-8 are depicted as mean ± SEM. *P < 0.001, **P < 0.001, ***$P < 0.001$ when comparing groups stimulated with LPS and exposed to HBO with groups not exposed to HBO using the Tukey test.

inflammatory cytokines (TNF-α, IL-6, and IL-8), and adhesion molecule (sICAM-1 and sVCAM) release after LPS stimulation compared to unstimulated samples (Groups A and B, *Tables 1* and *2*).

The same pattern was observed under HBO conditions concerning pro-inflammatory cytokines (TNF-α, IL-6, IL-8) after LPS stimulation compared to unstimulated samples (Groups C and D, *Table 1*). This increase under HBO exposure was significantly higher ($P < 0.001$) compared to normobaric conditions (Groups B and D, *Table 1*).

Conversely, HBO exposure alone did not affect TNF-α, IL-6, and IL- *8* levels, compared to normobaric conditions (Groups A and C, *Table 1*).

Also, there was a statistically significant decrease in sICAM-1 levels ($P < 0.001$) under HBO, post-LPS stimulation compared to normobaric conditions, while sVCAM levels remained almost invariable (Groups B and D, *Table 2*).

DISCUSSION

HBO treatment involves the inhalation of 100% O_2 at pressures greater than 1 ATA and is prescribed as an adjunctive therapy for conditions associated with gas emboli, compartment edema or ischemia and hypoxia. The increased PO_2 influences all tissues of the body by several different mechanisms [1], including the modulation of the immune response [2].

We proposed an experimental model based on whole blood sample stimulation, which has significant advantages [11] compared to *ex vivo* stimulation of isolated cells, even if it is well known, that in such experimental models there are a number of unpredictable and unknown variables, confusing the possible results. We used LPS as a potent stimulant of monocytes/macrophages that activates the pro- and anti-inflammatory cytokine cascade. Furthermore, pressures of 2.4 ATA have been widely applied as HBO treatment in clinical practice. Although patients often receive more than one HBO treatment, we have studied the effects of a single HBO exposure.

In our study, we found that pro-inflammatory cytokine release (TNF-α, IL-6, IL-8) was significantly increased after LPS stimulation. This effect was enhanced under hyperbaric conditions. However, we did not find any significant alterations of cytokine levels under HBO conditions, in the absence of LPS stimulation. These findings indicate an enhanced effect of HBO on the positive stimulatory effect of LPS on pro-inflammatory cytokine release.

There are a number of studies in the literature investigating the effects of HBO on the inflammatory response. Most of them used LPS-challenged animals and examined the effect of HBO on cytokine release from isolated cell populations of different tissues after exposure to HBO. An important methodological difference in our *ex vivo* experiments is that we examined the early (within four hours) inflammatory response of a previously activated immune response using LPS, under HBO conditions.

Firstly, Inamoto *et al.* [5] showed that IL-1 bioactivity was suppressed in mice, while IL-6 remained unchanged, under HBO conditions. They suggested a model using five consecutive exposures of the animals to HBO at 2.5 ATA for 60 min and investigated the effect of hyperbaric conditions on isolated, cultured, stimulated with LPS mouse splenic macrophages, and determined a radioactive - based bioactivity of IL-1 and IL-6. The long-term exposure and the additional incubations during cell culture could explain the reasons for the down-regulation of IL-1. It is well known that the inflammatory response involves the conversion of IL-1β to precursor IL-1β and the interaction between mature IL-1β and pre- IL-1β, at the post-translational level. This effect involves inhibiting the biological activity of IL-1β, although the decreased levels of IL-1 could be related to modifications of protein structure.

Table 2
HBO inhibits sICAM-1 but not sVCAM release, in whole blood (0.1 mL) from healthy individuals after LPS stimulation

Adhesion molecules (pg/mL)	Normobaric conditions		Hyperbaric conditions	
	Group A	Group B	Group C	Group D
sICAM-1	1 976 ± 183.6	4 421 ± 713*	1 879 ± 232	1 977 ± 153*
sVCAM	5 318 ± 224.6	6 198 ± 207.5**	6 829 ± 331	6 029 ± 283**

sICAM-1 and sVCAM are depicted as mean ± SEM. * $P < 0.001$, **$P > 0.05$ when comparing groups stimulated with LPS and exposed to HBO with groups not exposed to HBO using the Tukey test.

Lahat *et al.* [6] exposed normal rats to HBO 100% oxygen, at 2.8 ATA for 90 min once, and then isolated monocytes from the lungs, spleen and peripheral blood which were subsequently cultured for 18h. They found a spontaneous increase in TNF-α levels for cells from all tissues not stimulated with LPS after a single HBO exposure, compared to controls. On the other hand, LPS stimulation of samples after a single LPS exposure induced a similar increase in TNF-α levels that was however not significantly higher compared to spontaneous TNF-α release in samples exposed to HBO alone. The authors suggested that LPS stimulation induced a marked increase in TNF-α release compared to controls, but not after HBO exposure, and they concluded that TNF-α plays a key role in mediating the effects of HBO. However, we should note that these investigators used isolated cells and not whole blood or tissues, and they used an 18 h incubation period which was significantly than ours. These methodological differences may play a significant role in interpreting the results.

In patients with Crohn's disease IL-1, IL-6 and TNF-α levels were reduced during HBO treatment [12], as detected using endotoxin. Reduced TNF-α levels were also found by Luogo *et al.* [13] in a zymosan-induced shock model in rats.

Recently Benson *et al.* [7], based on Inamoto and Lahat findings [5,6], suggested an inhibitory effect of HBO on pro-inflammatory cytokine (IL-1β and TNF-α) release after stimulation, using multiple inducers, at the transcriptional and translational level. They designed an *in vitro* model based on isolated human mononuclear cells from human volunteers ($N = 4$-6), and on a single HBO exposure. They also kept exposed culture plates for 24 h at normobaric conditions. On the other hand, they focused on the effect of HBO on PHA-stimulated cells. PHA, which is an inducer of T cells and the cascade of released cytokines, is specific only for T-cells. These methodological differences as compared to our study, may explain the different results obtained.

In contrast to all previously mentioned studies, we terminated cultures immediately after HBO exposure for 90 min. This step and the short pre-induction time of whole blood with LPS, seem to be the main critical differences of our methodology. During the early steps of the inflammatory response, the expression of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8) is increased and is modified as the cascade of cytokines proceeds.

Our results are partly in accordance with the studies of Roco *et al.* [9] who found that serum TNF-α levels from healthy volunteers increased significantly during and after a single HBO session, pressurized at two and 2.8 ATA, while IL-6 and IL-8 did not change significantly. Ersson *et al.* [13] also studied cytokine release in rats, after acute hyperbaric exposure. They proved a post-dive increase in IL-6 levels, which may be the result of an activation of the inflammatory system by the creation of a blood-gas interface during exceedingly fast decompression. Furthermore, when administered after hemorrhage, HBO attenuates serum cytokine induction (IL-6 and TNF-α). This mechanism is thought to improve liver ischemia and tissue oxygenation [15].

We have also found that HBO exposure ameliorates the effect of LPS on sICAM-1 release, which is a very interesting observation. On the other hand, we found that LPS stimulation and HBO exposure alone enhances sVCAM release. We also found that HBO exposure attenuates sVCAM release in LPS-challenged samples as compared to LPS-challenged sample levels under normobaric conditions.

ICAM-1 is constitutively expressed by endothelial cells and is up-regulated rapidly during inflammation, resulting in increased leukocyte-endothelial cell adhesion. LPS and inflammatory cytokines, including TNF-α, IL-1 and IFN-γ stimulate ICAM-1 and VCAM mRNA accumulation and cell surface expression, although this mechanism is thought to promote tissue inflammation [3]. The up-regulation of gene expression of adhesion molecules in microvascular endothelial cells is an important step for the migration and accumulation of leukocytes at the site of inflammation, which play a critical role in organ damage during sepsis [16]. Increases in oxygen tension under normobaric but not hyperbaric conditions, stimulate expression of ICAM-1 and VCAM in human micro- and macrovascular endothelial cells, which may contribute to adhesion and transmigration of different leukocyte populations in I/R injuries. ICAM-1 has been implicated in sepsis-induced injury by recruiting DC11/CD18-expressing PMNs [17].

The ability of HBO to suppress the release of sICAM-1, as has been shown in the present study, may help explain the beneficial anti-inflammatory effect of HBO in treating I/R injury. The elevated levels of cytokines released under HBO post-LPS stimulation and especially IL-8, might mediate the decrease in sICAM-1.

The chemotactic cytokine IL-8 causes conformational changes in the structure of integrins. Integrins are adhesion molecules that serve as cell surface receptors for ICAMs and VCAMs [18, 19] on endothelial cells or lymphocytes. The higher levels of IL-8 released under HBO, mediating changes of adhesion molecules receptors structure, enhance the binding capacity of soluble ICAM-1 molecules, and the sICAM-1 levels detected were decreased. This mechanism is enhanced, as under HBO without stimulation, alterations of IL-8 or sICAM-1 levels observed were not significant. However, sVCAM remained unchanged under HBO, post-LPS. Perhaps binding of sVCAM to its receptor is a later process than sICAM-1.

The finding of a suppressive effect of HBO on sICAM-1 levels is in line with similar results reported by others. Treatment with HBO has shown some promising results in some models of ischemia, the major effect being a reduction in local ischemic injury. HBO has been shown to inhibit the adhesion of neutrophils to vascular endothelium and to decrease the overall PMN content within the ischemic tissue, following trauma [20]. Other studies using various animal models indicate that neutrophil recruitment is significantly reduced by HBO following I/R injury to the liver, intestine, and gracilis muscle [21-23].

Thom *et al.* [24] have shown that HBO reduces neutrophil adhesion to a variety of substrates including glass wool and fibrinogen-coated glass. Using neutralizing antibodies, the effect has been shown to be specific to the β_{2-} integrin class of neutrophil adhesion molecules, and is dose-dependent in the sense that pressures of at least 2.8 ATA for 45 min are needed for complete inhibition of adhesion. Recently, *in vivo* studies have shown that HBO treatment improves outcome following experimental I/R injury and that HBO effectively down-regulates ICAM-1 expression in an *in* *vitro* model of endothelial cell I/R injury, and this down-regulation may be mediated in part by endothelial nitric oxide synthase (eNOS) activity [25]. Kalns *et al.* [26] demonstrated that HBO specifically inhibits the various CD11B/CD18- (Mac-1) mediated neutrophil functions, in healthy human subjects. Larson *et al.* [27] have studied the effect of hyperbaric oxygen on neutrophil CD18 expression in an animal model of I/R injury. Their results suggest that HBO does not prevent the increase in CD18 expression.

In conclusion, exposure to HBO of unstimulated whole blood samples from healthy individuals, does not significantly affect cytokine release. Exposure to HBO of whole blood from healthy individuals previously challenged with LPS enhances cytokine release and suppresses sICAM-1 levels. As regards sVCAM levels, HBO exposure in samples previously challenged with LPS, resulted in sVCAM levels comparable to the LPS-challenged samples alone. These latest findings may support the potential beneficial effect of HBO on adhesion of PMNs to endothelium through modulation of ICAM-1 protein expression. Further investigations may allow a clearer understanding of the mechanisms by which HBO affects cytokine expression.

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