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Exposure to hyperbaric oxygen induces tumour necrosis factor-alpha (TNF-α) secretion from rat macrophages

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SUMMARY

We investigated the secretion of TNF-α by monocytes and macrophages derived from the peripheral blood, spleen, and lungs after a single exposure to a therapeutic profile of hyperbaric oxygen (HBO). Rats were exposed for 90 min to either 100% oxygen at 0·28 MPa (2·8 atmospheres absolute) or air. Immediately after exposure, mononuclear cells were isolated from blood, spleen, and lungs and cultured for 18 h. The secretion of TNF-α from the cultured monocytes/macrophages was determined with and without stimulation with lipopolysaccharide (LPS). Exposure to hyperbaric oxygen induced a significant increase in the spontaneous *ex vivo* secretion of TNF-α (without LPS) by mononuclear cells from the blood, spleen, and lung ($P < 0{\cdot}05$ from air controls). Stimulation with LPS after exposure to HBO induced a significant increase in TNF-α secretion by lung and spleen macrophages compared with air controls ($P < 0{\cdot}05$). However, absolute TNF-α levels were not significantly higher than those achieved 'spontaneously' in macrophages exposed to HBO without LPS. Stimulation with LPS induced a marked increase in secretion of TNF-α from blood monocytes after exposure to air, but not after exposure to HBO. These results provide evidence in support of a role played by TNF-α in mediating HBO effects on different tissues and their immune responses.

Keywords oxygen toxicity hyperoxia lipopolysaccharide immune system

INTRODUCTION

Hyperbaric oxygen (HBO) is commonly used in military and professional diving, and is well accepted as a treatment for decompression sickness, carbon monoxide intoxication, gas gangrene, air embolism, soft tissue infections, traumatic peripheral ischaemia, etc. [1,2]. Although beneficial, HBO may also be toxic and may affect immune responses, which are of the utmost importance in most of these conditions. Only limited information is currently available regarding the effect of a therapeutic hyperbaric oxygen profile on the immune system in normal and pathologic conditions [3–5]. In previous studies [6,7] we tested the effects of a single commonly used profile of exposure to hyperbaric oxygen on mononuclear cell subsets in healthy human volunteers and in an animal model. Our main findings were rapid post-exposure activation and redistribution of both monocytes and T cell subpopulations, with a significant reduction in the CD4/CD8 ratio in the blood and spleen and a concomitant increase in the lungs.

TNF-α, a major regulatory and effector cytokine, is secreted primarily by activated monocytes or macrophages [8]. It is characterized by multiple occasionally antagonistic actions. High TNF-α levels, sharply rising in a number of stress conditions, are deleterious both to the immune system and to the immediate clinical status [9,10], and have been implicated in the pathogenesis of widespread lung damage including adult respiratory distress syndrome (i.e. ARDS) [10] and oxygen toxicity [11]. On the other hand, pre-treatment with TNF-α protects rats against fatal pulmonary oxygen toxicity [11]. The precise role of TNF-α in the development of hyperoxic lung damage has not yet been established.

In most previous studies, exogenous TNF-α was used to explore its effects on the development of pulmonary oxygen toxicity [11–14]. We studied the effect of a single exposure to HBO on the endogenous secretion of TNF-α by blood monocytes, by macrophages from the lungs (as the main target of the oxidative insult) and by macrophages of the spleen (as a major immunoregulatory organ). The cells were cultured without additional triggering to reflect the response to *in vivo* induction of TNF-α (*'ex vivo'*), and with lipopolysaccharide (LPS) to assess the ability of the *in vivo* exposed cells to sustain the TNF-α response after a further immunological challenge.

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MATERIALS AND METHODS

Experiments were carried out on male Sprague Dawley rats weighing 220–280 g, fed *ad libitum* on commercial food and kept in a normal day/night cycle.

Experimental protocol

Sixteen rats were randomly assigned to two groups exposed to either 100% oxygen at 0·28 MPa or air for 90 min. Exposure to HBO was carried out in a 3-*l* Plexiglas cage placed inside a 150-*l* animal hyperbaric chamber (T.C.A.H.O., Technical Centre for the Applications of Hyperbaric Oxygen, Roberto Galeazzi, Italy). The small Plexiglas cage was flushed with oxygen, while the pressure in the large chamber was maintained with compressed air. A constant gas flow of 2 *l*/min was employed to prevent the accumulation of carbon dioxide in the Plexiglas cage. Chamber temperature was maintained at 22°C, with a transient rise in temperature of up to 2°C during or shortly after compression, which was carried out at the rate of 0·1 MPa/min. A group of control rats was exposed to air at atmospheric pressure in the same hyperbaric chamber, and subjected to the same handling, noise and gas flow procedures, etc., to preclude differences in the level of stress. Immediately after the end of the exposure, the rats were anaesthetized by pentobarbital.

Isolation of peripheral blood mononulear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood drawn from the right atrium, by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density gradient and centrifugation for 25 min at 700 ***g***. The band of mononuclear cells was suspended in PBS, and washed twice at 220 ***g*** for 10 min. The cells were washed again, suspended in an LPS-free RPMI 1640 + 10% fetal calf serum (FCS) medium and counted.

Isolation of mononuclear cells from the spleen

The spleen was removed, suspended in PBS, mashed in a sterile 50 mesh metal net, and centrifuged for 8 min at 220 ***g***. Erythrocytes were removed by hypotonic solution (distilled water for 15 s), after which the lymphocytes were centrifuged twice in PBS, suspended in an RPMI 1640 medium, and counted.

Isolation of mononuclear cells from the lung

The lung vascular bed was flushed with 20 ml RPMI 1640 introduced via the right atrium of the heart. Washing was continued until the lungs were clear white, at which point they were immediately excised. The lungs were sliced to a thickness of 500 μm using a Mcllwain tissue chopper, and then separated by teasing and suspended for 15 min in 30 ml RPMI 1640 and 5 mg/ml dispase (Sigma, St Louis, MO) in a shaking water bath at 37°C. Any remaining intact tissue was suspended for 20 min in 30 ml RPMI 1640 containing 1 mg/ml collagenase IV (Sigma) in a shaking water bath at 37°C. The cells in the supernatants were collected, centrifuged, and suspended in RPMI 1640 + 10% FCS. Collagenase was added, and the procedure was repeated up to four times. All isolated cells were loaded onto the Ficoll–Hypaque and centrifuged for 25 min at 700 ***g***. The band of mononuclear cells was washed twice for 10 min at 220 ***g***, and the cells were suspended in LPS-free RPMI 1640 + 10% FCS + 1% antibiotics and counted.

Isolation and culture of monocytes/macrophages

Mononuclear cells derived from the blood, spleen and lungs were cultured in a 'complete medium' containing RPMI 1640, 1% antibiotics, 5×10^{-5} M mercaptoethanol, 1% non-essential amino acids, and 1% pyruvate. Cells (2×10^6 cells/well per ml) were incubated in 24-well plates for 90 min (5% CO_2 at 37°C), and then the floating non-adherent cells were removed by washing the wells twice. The identity of monocytes/macrophages was verified using FITC anti-rat CD11b/c antibodies (Pharmingen, San Diego, CA). Of the adherent cells, 75–90% were positive by FACS analysis. Adherent cells were then cultured in a 'complete medium' with or without LPS (1 μg/ml). This monocyte/macrophage-enriched population was incubated for 18 h in a 37°C incubator. Supernatants were then removed into microtitre wells and kept frozen (−80°C) until assayed for TNF-α content.

TNF-α assay

TNF-α was measured according to Meager *et al.* [15]. L-929 fibroblast cells at a concentration of 5×10^3 cells/well were plated in 96-well, flat-bottomed plates. When the monolayer became confluent, 4 μg/ml of actinomycin D (Sigma), together with either recombinant TNF-α or experimental supernatant, were added. Murine recombinant TNF-α standards with known concentrations (pg/ml) were derived from a murine TNF-α kit (Genzyme, Cambridge, MA) and served as the reference reagents in the bio-assay kit. After incubation for 24 h, 100 μg/well of MTT (M2128; Sigma) were added with subsequent incubation for 4 h at 37°C. Following gentle aspiration of the supernatant, 0·1 ml of isopropanol–HCl 0·04 N was added to each well and shaken vigorously for 15 min. The absorbance at 570 nm (with reference 630 nm) was then read, and measurements for the dilution series were plotted to produce dose–response curves on which the OD read was proportional to the reciprocal of the TNF-α concentration. Neutralizing hamster anti-murine TNF-α MoAbs reacting with rat TNF-α (Genzyme) [16] were incubated with several supernatants in which high TNF-α values were measured in the first assay. When these supernatants were tested in a second identical TNF-α assay, they lost their activity, thus confirming the specificity of the TNF-α assay (data not shown).

Wet-to-dry weight ratio of the lung

Two additional groups, each comprising five rats, were subjected to the same two experimental protocols of exposure to HBO or air with removal of the lungs after 90 min. The wet weight of the lungs was measured immediately. The dry weight was determined after 24 h in a lyophilizer, for calculation of the wet-to-dry lung weight ratio.

Statistical analysis

The experimental results for the two groups of rats exosed to HBO or air were compared by the Mann–Whitney test and expressed as mean ± s.e.m. Comparison between data means within each group of rats exposed to air or oxygen was done by Wilcoxon signed rank test.

RESULTS

Figure 1 illustrates the 'spontaneous' secretion of TNF-α by cultured monocytes/macrophages derived from the blood,

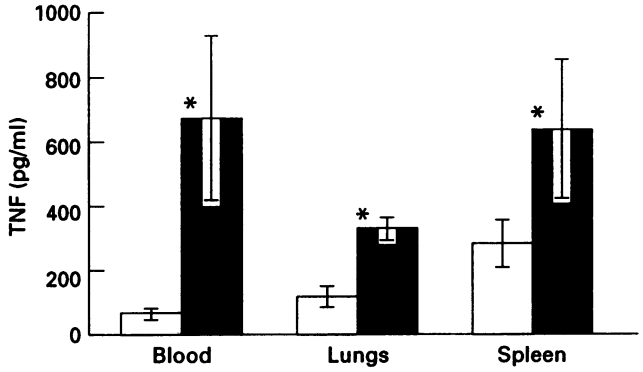

Fig. 1. Untriggered secretion of TNF by cultured monocytes/macrophages derived from peripheral blood, lung, and spleen of rats exposed to hyperbaric oxygen (HBO) (■) or air (□). All values are mean ± s.e.m. *TNF values in the group exposed to HBO significantly higher than in the group exposed to air ($P < 0.05$).

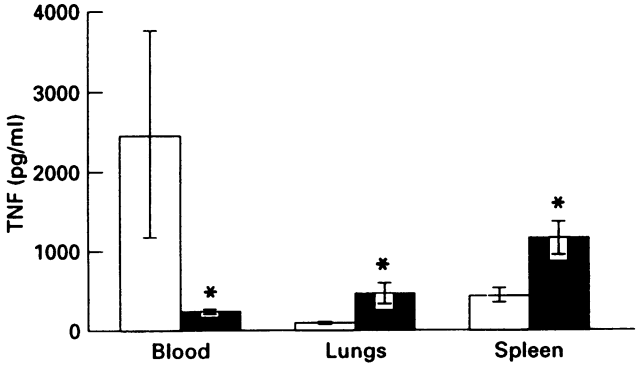

Fig. 2. Secretion of TNF by cultured monocytes/macrophages derived from peripheral blood, lung, and spleen of rats exposed to hyperbaric oxygen (HBO) (■) or air (□) after *in vitro* triggering with lipopolysaccharide (LPS). All values are mean±s.e.m. *TNF values in the group exposed to HBO significantly higher than in the group exposed to air ($P < 0.05$); ** TNF values in the group exposed to HBO significantly lower than in the group exposed to air ($P < 0.05$).

lungs, and spleen of rats exposed to HBO or air. When cultured without LPS, TNF-α secretion reflects the response of the cells to previous *in vivo* triggering, together with the possible stimuli evoked by the isolation and culture techniques ('*ex vivo*'). The difference between the two groups may therefore be attributed to the exposure to oxygen at high pressure. As shown in Fig. 1, significantly higher *ex vivo* secretion of TNF-α was observed in monocytes/macrophages derived from the blood, spleen, and lungs of rats exposed to HBO compared with air controls.

The ability of monocytes or macrophages derived from the three tissues to secrete TNF-α after the addition of LPS is depicted in Fig. 2. A significant increase in TNF-α was observed in lung and spleen macrophages exposed to HBO *in vivo*, compared with the corresponding macrophages derived from rats exposed to air. In contrast, LPS triggering of peripheral blood monocytes after *in vivo* exposure to HBO induced significantly attenuated secretion of TNF-α compared with air controls.

We also compared the effect of LPS triggering on absolute values of TNF-α secretion within each group of monocytes or macrophages (HBO or air). In both groups, LPS increased the secretion of TNF-α from lung and spleen macrophages. However, the differences did not reach statistical significance. Monocytes derived from peripheral blood behaved differently. When these cells were exposed to air *in vivo*, minimal 'spontaneous' secretion of TNF-α was detected. The addition of LPS induced a marked increase in their ability to secrete TNF-α ($P < 0.02$). In contrast, after exposure to HBO, peripheral blood monocytes exhibited 'spontaneous' TNF-α secretion which was not altered significantly by the addition of LPS.

There was no significant difference ($P = 0.4$) between the wet-to-dry lung weight ratio of rats exposed to HBO and air controls (4·5 + 0·09 and 4·6 + 0·07, respectively).

DISCUSSION

Our study demonstrates that a single exposure to therapeutic profile of HBO induced secretion of TNF-α from rat blood monocytes and macrophages derived from spleen and lungs. Moreover, the exposure also changed the response of monocytes/macrophages to further *in vitro* stimulation with LPS. In a recent study [12] performed by Jensen *et al.*, an enhanced level of TNF-α mRNA was found in the lungs of mice after prolonged toxic exposure to normobaric hyperoxia (> 95% at 1 atmosphere absolute for 3 days), without detectable serum TNF-α activity. The difference between our study and that of Jensen *et al.* could be related to the ability of oxygen to trigger local rather than systemic changes in TNF-α secretion, to our use of specific isolated cell populations, to our culture manipulations which could have further enhanced translation of existent TNF-α mRNA, or to the higher dose of oxygen employed in our protocol.

TNF-α has been implicated in the pathogenesis of pulmonary toxicity, as its administration caused pulmonary changes similar to those of ARDS and of pulmonary oxygen toxicity [17]. Increased endogenous production of TNF-α has also been linked to lung injuries associated with bleomycin and silica [18,19]. These conditions are related to increased production of reactive oxygen species, and could be ameliorated in experimental animals by the administration of antibodies to TNF-α [18,19]. Furthermore, treatment with an antibody to TNF-α improved the survival of mice exposed to prolonged normobaric hyperoxia [12]. On the other hand, it has been shown that pretreatment with TNF-α protects against lethal pulmonary oxygen toxicity [11,12,20], possibly through induction of manganese superoxide dismutase (MnSOD) in lung epithelial and endothelial cells, and a decrease in microsomal production of reactive oxygen metabolites [14,20,21]. Moreover, it has been found that administration of TNF-α to the lung contributed to host defence mechanisms, since it increased the production of IL-1 by pulmonary and blood macrophages/monocytes, enhanced their ability to lyse tumour cells and elevated their surface Ia expression [22]. Thus TNF-α plays a dual action in the pathogenesis of hyperoxic lung damage, depending on its tissue and systemic levels, the concentration of other cytokines, and the exact timing of its administration or endogenous production. It should be emphasized that the exposure profile employed in our study, a high partial pressure of oxygen for a short time, does not induce pulmonary toxicity or any sign of respiratory distress, and is considered safe [23]. The role of the TNF-α secreted in response to this profile of HBO exposure is

not clear. It could be either part of a defence mechanism against the deleterious effects of oxygen, or it could initiate the early subclinical phase of the pathophysiological sequence of events leading to pulmonary oxygen toxicity.

The experiments in which exogenous LPS was added to the cell cultures were designed to evaluate the effect of HBO on the ability of monocytes and macrophages to respond to a new immunological challenge. We found that LPS was not able to mount significant TNF-α secretion from both HBO pre-exposed and control lung and spleen macrophages over that observed '*ex vivo*'. Therefore at least *in vitro* LPS does not seem to be a major trigger for TNF-α secretion from these tissue macrophages. Following culture with LPS, peripheral blood monocytes derived from control rats secreted high amounts of TNF-α, in agreement with previous publications [24–26]. On the other hand, HBO-pre-exposed monocytes triggered with LPS had reduced ability to secrete TNF-α. The divergent response of blood monocytes and tissue macrophages is not surprising, since monocytes and specialized tissue macrophages differ in their differentiation state, physiological functions and immunological responses [27]. Moreover, stimulatory and inhibitory effects of exposure to oxygen on different or similar mononuclear cell subsets from different tissues have already been observed and depend upon the cell subsets, the tissue and its specific oxygen requirements, and the duration of exposure to oxygen [28,29]. Thus, inhibition of TNF-α secretion could be caused by TNF-α itself [30], or by other factors which induce exhaustion of the TNF-α synthesizing machinery [31–33]. Pre-exposure to endotoxin has recently been found to inhibit TNF-α release after a second endotoxin stimulus [34]. The nature of the trigger for HBO-induced TNF-α production and the role of endotoxin (LPS), if any, remain to be evaluated.

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