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Hyperbaric oxygen increases plasma exudation in rat trachea: involvement of nitric oxide

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1 This study investigates the microvascular permeability changes in tracheal tissue of rats exposed to hyperbaric oxygen (HBO).

2 Rats, following exposure to HBO or ambient air (control animals) for 1.5, 3 and 6 h, were prepared for recording of nitric oxide exhaled (FENO) in air using a chemiluminescence analyser. The level of FENO was not statistically different in the two groups. Plasma exudation, evaluated by measuring the leakage of Evans blue (EB) dye into the tracheal tissue, was significantly elevated (48, 86 and 105% at 1.5, 3 and 6 h, respectively) in HBO-treated rats.

3 Plasma exudation in the trachea of control rats was significantly increased (42%, $P<0.05$) by N^G-nitro-L-arginine methyl ester (L-NAME), whereas it was significantly reduced (31%, $P<0.05$) in rats exposed to HBO for 3 h.

4 N-acetylcysteine (NAC) and flunisolide significantly prevented the increase in plasma leakage in HBO-treated rats. In contrast, indomethacin was devoid of anti-exudative activity in these experiments.

5 Western immunoblot showed a significant increase in the level of inducible nitric oxide synthase (iNOS) protein in the tracheal homogenates of HBO-treated rats, as compared to basal levels.

6 These results indicate that nitric oxide (NO) is involved in the maintenance of microvascular permeability in tracheal tissue of rats. The protective effect observed with the steroid seems to support this hypothesis. Furthermore, the beneficial action of NAC underlines that reactive oxygen species participate in the microvascular permeability changes observed in tracheal tissue of rats exposed to HBO.

Keywords: Hyperoxia; microvascular permeability; expired nitric oxide (FENO); oxygen free radicals; L-NAME; flunisolide; indomethacin; N-acetylcysteine

Abbreviations: ATA, atmospheres absolute pressure; cNOS, constitutive nitric oxide synthase; EB, Evans blue; FENO, fractional expired nitric oxide; HR, heart rate; HBO, hyperbaric oxygen; iNOS, inducible nitric oxide synthase; IFNγ, interferon gamma; LPS, lipopolysaccaride; MABP, mean arterial blood pressure; NAC, N-acetylcysteine; D/L-NAME, N^G-nitro-D/L-arginine methyl ester; p.p.b, parts per billion; p.p.m., parts per million; ROS, reactive oxygen species

Introduction

It is known that the lungs are particularly sensitive to oxidant damage during prolonged exposure to environments with increased oxygen content at 1 a.t.m. (normobaric hyperoxia) or when the exposure to pure oxygen is carried out at more than 1 a.t.m. (hyperbaric oxygen) and for several days. In fact, alterations in lung structure and function such as tissue and alveolar oedema, surfactant dysfunction, lung inflammation and decreased pulmonary compliance have been reported (Amin *et al*., 1993; Jenkinson, 1993). In recent years, an increasing number of patients has been subjected to HBO therapy in order to supplement blood oxygen content to control different types of ischaemic organ damage with particular reference to wound healing problems and infectious diseases (Amin *et al*., 1993). However, increased oxygen ambient pressure is a significant factor that could aggravate normobaric pulmonary oxygen toxicity and lead to the development of chronic lung disease or death. Although the exact mechanisms of pulmonary oxygen toxicity are unknown, Gerschman (1964) first suggested that oxygen toxicity may be mediated in large part by the production of reactive oxygen species (ROS) that act through peroxidation of membrane lipids, oxidation of membrane proteins and breakage of DNA strands (Wispè & Roberts, 1987). Acute HBO exposure of rats markedly alters pulmonary vascular responses following extravascular fluid accumulation (Amin *et al*., 1993). In rabbits, HBO treatment for 1 h causes marked pulmonary hypertension and lung weight gain (Jacobson *et al*., 1992). In a recent study by our group (Radice *et al*., 1997) we reported an impairment of vascular endothelium-dependent relaxant mechanisms in coronary artery of isolated hearts obtained from HBO exposed rats. This event, associated with a worsening of myocardial ischaemia-reperfusion damage, was attributed to increased free radicals formation and was prevented by N-acetylcysteine (Rossoni *et al*., 1997).

Nitric oxide (NO) is a potent autacoid formed from L-arginine and molecular oxygen by the constitutive nitric oxide synthase (cNOS) in a variety of cells, particularly pulmonary vascular endothelium and human lung epithelial cells (Szabò 1995; Robbins *et al*., 1994). In addition, an inducible NO synthase (iNOS) is expressed in different models of inflammation (Vane *et al*., 1994; Tomlinson *et al*., 1994; Hutcheson *et al*., 1990). In rats, lipopolysaccaride (LPS) treatment results in an increased expression of iNOS mRNA in homogenates of whole lung (Liu *et al*., 1994) or in iNOS induction in tracheal tissue (Bernareggi *et al*., 1997). Exposure of mice to normobaric hyperoxia has been demonstrated to be associated with a significant increase in NO production, measured as total

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nitrite and nitrate in bronchoalveolar lavage fluid (Arkovitz *et al*., 1997). Oxidant stress, as well, has recently been shown to induce iNOS in pulmonary alveolar epithelial cells (Adcock *et al*., 1994).

Plasma exudation in the tracheaeobronchial airways represents a mucosal defence mechanism. However, under certain conditions, plasma leakage from tracheobronchial vessels may also result in inflammatory consequences that are important in airway disease (Persson, 1986). In fact, it has been demonstrated that when NO is constitutively present in the airways it may play a protective role, but when iNOS is expressed the increased production of NO may be responsible of deleterious effects. In fact, the NOS inhibitor, L-NAME, increases plasma leakage into the trachea in rat airways, whereas it inhibits LPS-induced vascular damage (Bernareggi *et al*., 1997).

Although several studies have demonstrated that the lungs are particularly susceptible to oxidant injury during acute or prolonged exposure to hyperbaric oxygen (Amin *et al*., 1993; Jacobson *et al*., 1992), there is limited information regarding what contribution NO and ROS may have on plasma leakage in the large airways. We have, therefore, examined the relationship between acute HBO exposure and the consequent microvascular leakage in the tracheal tissue of rats. In addition, we have investigated the relevance of NO and ROS formations to the HBO-induced plasma exudation in the airway tissues.

Methods

Hyperbaric oxygen treatment

Male albino rats of the Sprague-Dawley strain (Charles River Italia, Calco, CO, Italy), weighing 250–300 g were introduced (two animals each time) into a small (25 cm diameter, 50 cm long) hyperbaric chamber with one compartment (Sistemi Iperbarici Integrati S.p.A., Rome, Italy) and exposed for a period of 1.5, 3 and 6 h to HBO (100% oxygen; 2.5 atmospheres absolute pressure, ATA). In order to eliminate carbon dioxide accumulation, the chamber was flushed with 100% oxygen for 1 min every 30 min during exposure. All experimental protocols were approved by the Review Committee of the Department of Pharmacology and met the Italian guidelines for laboratory animals which conform with the European Communities Directive of November 1986 (86/609/EEC).

At the end of HBO or ambient air exposure, the animals were anaesthetized with pentobarbitone sodium (60 mg kg^{-1} i.p.) and prepared for recording of systemic blood pressure and heart rate by placing a catheter in the right carotid artery. The trachea was cannulated for mechanical ventilation with synthetic ultrapure air (NO-free air, Air SP, Sapio, Monza, MI, Italy) performed by a pump (mod. 29488, U. Basile, Comerio, VA, Italy) operating on a partially closed circuit (10 ml kg^{-1} stroke volume; 70 cycles min^{-1}). To avoid spontaneous breathing, the animals were treated with pancuronium bromide injected *via* the jugular vein at a dose of 1 mg kg^{-1} and heparin (10 UI kg^{-1}) was then administered intravenously. Changes in blood pressure were measured by pressure transducers (mod. 7016 U. Basile) and signals displayed on a two-channel pen recorder (mod. Gemini, U. Basile).

After completion of the surgical procedure, expired NO detection and vascular permeability measurements were carried out in less than 20 min. Due to the short duration of the experiment, the dose of pentobarbitone sodium used was sufficient to maintain a deep anaesthesia throughout the protocol.

Expired nitric oxide measurements

Fractional expired NO (FENO) was continuously measured using a chemiluminescence analyser (Model 280A; Sievers, Boulder, CO). This device uses ozone to oxidize NO to NO_2 in an excited state. Light is emitted when transition of NO_2 from excited to ground state occurs. The detection limit of the NO analyser is <1 p.p.b. with a repeatability of ± 1 p.p.b over a linear range $<1-500{,}000$ p.p.b. The response time is 200 ms. The analyser was designed for on-line recording of simultaneous measurements of the gas. This feature obviates the need for collection in a reservoir, with its variable loss of reactive NO and gives greater sensitivity and reproducibility (Kharitonov *et al*., 1994). A certified standard mixture of 100.1 p.p.m. NO in nitrogen (N_2) was used to calibrate the instrument and linearity was checked by serial dilution with N_2. Ambient air NO concentration was recorded and the absolute zero was adjusted before each measurement by flushing the NO analyser with NO-free certified compressed air. The instrument permitted digital conversion of the analogue signals for data storage and off-line analysis. Gas was drawn continuously from a teflon catheter positioned within the tracheostomy tube and recorded on a breath by breath basis. The peaks average concentration of NO at end expiration over 70 ± 1 breaths was used as the FENO concentration (expressed as p.p.b.).

Vascular permeability measurements

Protein leakage, a marker of vascular permeability, was evaluated by measuring the leakage of Evans blue (EB) dye (Belvisi *et al*., 1989) into the tracheal tissue. This method has been previously shown to correlate well with the leakage of radiolabelled albumin (Rogers *et al*., 1989) in guinea-pig airways. Specifically, the EB dye (20 mg kg^{-1}) was injected in the jugular vein at time 0 and its tissue content was determined 5 min after the injection by perfusing the systemic circulation with saline to remove intravascular dye. Specifically, the left ventricle was incised, a blunt ended needle inserted into the aorta and the ventricles cross-clamped. Blood was expelled from the incised right atrium at 100 mmHg pressure until the perfusate was clear (125 ml infused). The trachea was removed, blotted dry and weighed. EB dye was extracted in formamide at 37°C for 18 h and its concentration was determined with a spectrophotometer (Shimadzu UV-160A Shimadzu, Tokyo, Japan) at the absorbance maximum of 620 nm wavelength. The tissue content of the EB dye (ng EB mg^{-1} wet wt. tissue) was calculated from a standard curve of EB dye concentration in the range of $0.12-20$ μg ml^{-1}.

Experimental protocol

Effect of HBO treatment on time-dependent plasma leakage in rat trachea After exposing rats to HBO or ambient air for 1.5, 3 and 6 h, EB dye (20 mg kg^{-1} i.v.) was injected *via* a jugular vein and plasma leakage was determined. The results obtained from this experiment allowed us to choose the optimal time of HBO treatment for use in the following experiments.

Effect of L-NAME, D-NAME and flunisolide on plasma leakage into the trachea of control and HBO-treated rats To investigate the role of NO in plasma leakage into the rat

trachea under control and HBO-treated conditions, L-NAME (100 mg kg^{-1} i.p.), an inhibitor of both constitutive and inducible isoforms of NOS (Rees *et al*., 1990), D-NAME (100 mg kg^{-1} i.p.), its inactive enantiomer, and isotonic sterile saline (1 ml kg^{-1} i.p.) were injected 1 h before exposure to ambient air (3 h) or HBO (3 h).

In other experiments, rats were pre-treated for 1 h prior to either ambient air exposure (3 h) or HBO exposure (3 h) with flunisolide (1 mg kg^{-1} i.p.) or its vehicle (1 ml kg^{-1} i.p.).

Effect of N-acetylcysteine and indomethacin on plasma leakage into the trachea of control and HBO-treated rats To investigate the potential contribution of oxygen free radicals to the increase in vascular permeability observed in rat trachea after HBO treatment, animals received NAC (1 g kg^{-1} p.o.) or isotonic sterile saline (1 ml kg^{-1} p.o.) once a day for 2 days and immediately before exposure to ambient air (3 h) or HBO (3 h).

To assess the relative contribution of prostanoids to the increased plasma leakage observed in trachea after HBO treatment, rats were treated with indomethacin (5 mg kg^{-1} i.p.) or isotonic sterile saline (1 ml kg^{-1} i.p.) 30 min before exposure to ambient air (3 h) or HBO (3 h).

Western immunoblot analysis

In separate experiments, iNOS protein expression was measured in the trachea of six rats exposed to HBO or ambient air for 3 h. At the end of exposure, the animals were killed by cervical dislocation, tracheal tissue was then removed and homogenates from tracheal tissues were centrifuged at 5000 × *g* for 15 min at 4°C. Proteins were boiled (10 min) in gel loading buffer in a ratio of 1 : 1 (v/v) and a determined amount (10 μg) was subjected to electrophoresis on sodium dodecyl sulphate 10% polyacrylamide gels according to the method of Maizel (1979). Western immunoblot analysis was performed as described by Towbin *et al*. (1979). The separated proteins were transferred electrophoretically from the polyacrylamide gel to nitrocellulose sheet in a blotting buffer (25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3), using a mini-transblot apparatus. The proteins were transferred onto nitrocellulose membrane. The membrane was saturated by incubation at 4°C overnight with Marvell (powered milk) solution (5% Marvell, 0.05% Tween 20) and then primed with a primary polyclonal anti-iNOS. The primary antibody was located with an alkaline phosphatase-conjugated second antibody with BCIP (5-bromo-4-chloro-3-indoylphosphate p-toluidine salt) and NTB (p-nitro blue tetrazolium chloride) colour development reagents. All antibodies were used at 1 : 1000 diluition.

Materials

The following drugs were used: formamide (Merck, Darstad, FRG); Evans blue, indomethacin, N^G-nitro-D/L-arginine methyl ester (Sigma Chem. Co., St. Louis, MO, U.S.A); pancuronium bromide (N.V. Organon, Oss, The Netherlands); N-acetylcysteine (Zambon, Italia srl, Bresso, Milano, Italy); flunisolide idrate (Valeas SpA, Milan, Italy); pentobarbital sodium (Abbott SpA, Campoverde di Aprilia, LT, Italy); heparin (Squibb, LT, Italy); anti-iNOS antibody (Transduction Laboratories, Lexington, U.K.).

Statistics

Results are expressed as the mean ± s.e.mean of *n* animals. In all experiments, the differences between control and treatment groups were analysed for statistical significance using a one-way analysis of variance (ANOVA) and Student's two tailed *t*-test for paired or unpaired samples as appropriate; $P<0.05$ was accepted as significant.

Results

Effect of HBO treatment on haemodynamic parameters

The exposure of rats to HBO regimen (1.5, 3 and 6 h) did not induce a status of aggressivity to handling and did not modify the haemodynamic parameters when measured in anaesthetized animals. In fact, baseline values for MABP and HR of control rats (147 ± 4 mmHg; $n=18$; 418 ± 8 b min^{-1}; $n=18$) were not significantly different ($P>0.05$) from those of rats exposed to HBO (144 ± 4 mmHg; $n=18$; 400 ± 9 b min^{-1}; $n=18$). These parameters were constant throughout the duration of the experiment.

Effect of HBO treatment on FENO concentrations

The HBO treatment did not provide any statistical modification of the basal concentration of FENO when compared to that in the expired gas of control rats ($P>0.05$). In fact, the basal FENO values in the control rats were 5.0 ± 0.81 p.p.b ($n=13$) and in HBO exposed rats were 5.0 ± 0.75 p.p.b ($n=14$).

Effect of HBO treatment on plasma leakage in rat trachea

Figure 1 shows plasma leakage in tracheal tissue of control and HBO-treated rats at 1.5, 3 and 6 h. HBO exposure caused a significant increase in plasma leakage of 48, 86 and 105% in HBO exposed animals compared with controls at 1.5, 3 and 6 h exposure, respectively. Since the HBO treatment at 3 h was significantly different from 1.5 h ($P<0.05$) but not from 6 h, a 3 h exposure was used in the following experiments.

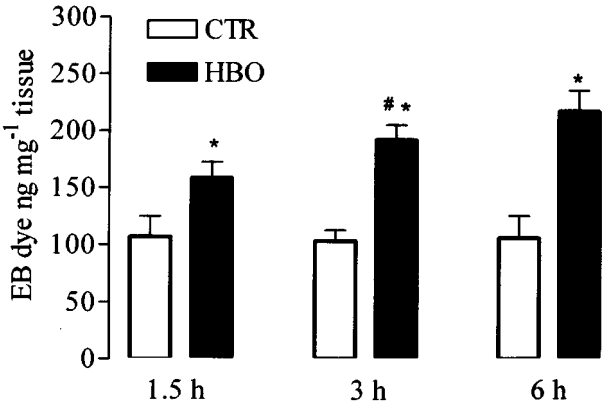

Figure 1 Effect of duration of exposure to hyperbaric oxygen on plasma leakage in rat tracheal tissue. Rats were exposed to hyperbaric oxygen (HBO, 100% oxygen; 2.5 ATA) treatment or ambient air for 1.5, 3 and 6 h. At the end of the exposure rats were anaesthetized and protein leakage was evaluated by measuring the leakage of Evans blue (EB) dye into the tracheal tissue. Columns represent the mean value ± s.e.mean of six experiments at each time point. CTR, rats not exposed to HBO; HBO, rats exposed to hyperbaric oxygen. Significant differences between control and HBO-treated groups at each time points are demonstrated as $*P<0.05$ according to one-way ANOVA. Significant differences between 1.5 and 3 h HBO-treated rats are demonstrated as # $P<0.05$ according to one-way ANOVA.

Effect of L-NAME, D-NAME and flunisolide on tracheal plasma leakage in control and HBO-treated rats

In the trachea of rats exposed for 3 h to ambient air L-NAME significantly increased plasma leakage by 42% ($P<0.05$). In contrast, L-NAME significantly inhibited the HBO-induced plasma leakage in the trachea by 31% ($P<0.05$) (Figure 2). D-NAME did not modify EB dye leakage in the trachea of control (vehicle for D-NAME, 99.5 ± 6.8 ng mg^{-1}, $n=4$; D-NAME-treated, 108.3 ± 19.3 ng mg^{-1}, $n=6$, $P>0.05$) and HBO-treated rats (vehicle for D-NAME, 195.8 ± 10.5 ng mg^{-1}, $n=4$; D-NAME-treated, 187.4 ± 19.3 ng mg^{-1}, $n=6$, $P>0.05$).

In the trachea of HBO-treated rats, flunisolide inhibited plasma leakage by 41% ($P<0.05$) so that the leakage resembled that found in vehicle for flunisolide-treated control rats. Flunisolide had no effect on leakage in control rats (Figure 3).

Effect of N-acetylcysteine and indomethacin on tracheal plasma leakage in control and HBO-treated rats

In the trachea of HBO-treated rats, NAC significantly inhibited plasma leakage by 42% ($P<0.05$) so that the phenomenom resembled that found in vehicle for NAC-treated control rats. NAC had no effect on tracheal leakage in control rats (Figure 4).

Indomethacin had no effect on plasma leakage in the trachea of either control rats (vehicle for indomethacin, 100.4 ± 6.5 ng mg^{-1}, $n=4$; indomethacin-treated, 116.7 ± 16.8 ng mg^{-1}, $n=6$, $P>0.05$) or HBO-treated rats (vehicle for indomethacin, 182.8 ± 13.5 ng mg^{-1}, $n=4$; indomethacin-treated, 171.9 ± 20.2 ng mg^{-1}, $n=6$, $P>0.05$).

Western immunoblot analysis

The levels of iNOS protein from tracheal homogenates of rats exposed for 3 h to HBO were investigated by Western immunoblot analysis. Low levels of iNOS protein expression were detectable in control rats (lane b). The 3 h exposure to HBO resulted in an increase of iNOS protein expression (lane c) (Figure 5).

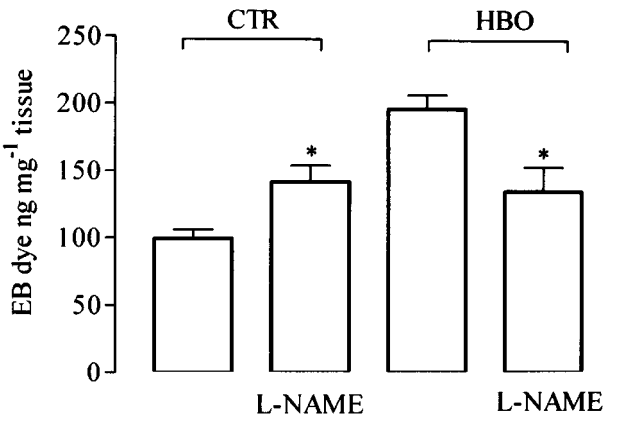

Figure 2 Effect of L-NAME on plasma leakage of tracheal tissue in control and HBO-treated rats. Rats were treated with L-NAME (100 mg kg^{-1} i.p.) 1 h prior to exposure to HBO or ambient air for 3 h. Columns represent the mean value$\pm$s.e.mean of 4–6 experiments. CTR, rats not exposed to HBO for 3 h. HBO, rats exposed to hyperbaric oxygen for 3 h. Significant differences between L-NAME-treated and untreated rats in both control and HBO-treated groups are demonstrated as $*P<0.05$ according to one-way ANOVA.

Discussion

Pulmonary oxygen toxicity is the primary limiting factor in the therapeutic administration of oxygen (Winter *et al*., 1972; Jenkinson, 1993). The diffuse cellular infiltration of lungs, proteinaceous exudate and consequent hypoxemia concurrent with high inspired concentration of O_2 has been shown to be harmful (Vacchiano & Tempel, 1994).

The present results clearly demonstrate that the microvascular permeability of the large airways of rats is increased when the animals are exposed for a limited period of time (3 h) to HBO. The mechanism/s involved in this phenomenon is difficult to understand. However a large contribution of free radicals appears a reasonable hypothesis. In fact, neutrophilis, macrophages and vascular endothelial cells, all found in abundance in airways, can produce a variety of free radicals when appropriately stimulated (Radi *et al*., 1991). Rat lung homogenates and mitochondrial particles exposed to high oxygen concentration showed increased superoxide anion (O^{-}_{2}) and hydrogen peroxide (H_2O_2) production. In addition, increased lipid peroxidation in the presence of hyperoxia has been reported (Freeman *et al*., 1982; Turrens *et al*., 1982). The fact that NAC administration to rats prior to HBO exposure abolished the increase in plasma exudate indicates for a large contribution of free radicals in the permeability changes of the tracheal vasculature. Beneficial effects of NAC in several ischaemia reperfusion models have been mostly referred not only to its antioxidant activity but also to its direct scavenging action on hydroxyl radicals (Villa & Ghezzi, 1995; Brunet *et al*., 1995).

Another finding emerging from the present experiments refers to the opposite effect on plasma exudation observed in rats treated with the inhibitor of NO synthase, L-NAME. This compound given to control rats brings about a significant increase in plasma extravasation in tracheal tissue, whereas it inhibits this event in rats exposed to HBO. The demonstration that in the trachea under 'physiological' conditions NOS

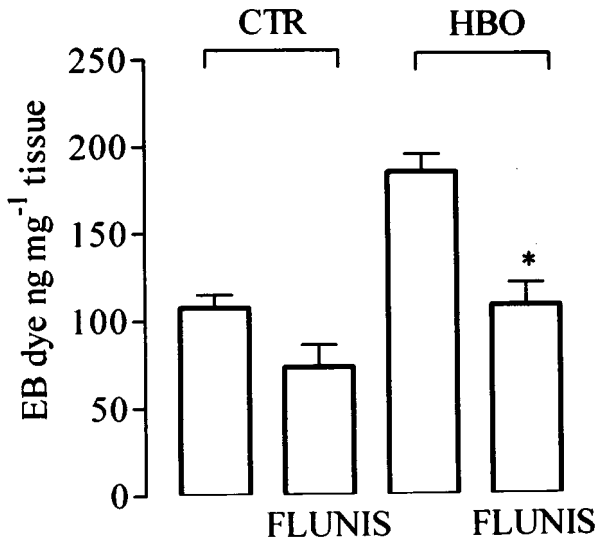

Figure 3 Effect of flunisolide on plasma leakage of tracheal tissue in control and HBO-treated rats. Rats were treated with flunisolide (FLUNIS, 1 mg kg^{-1} i.p.) 1 h prior to exposure to HBO or ambient air for 3 h. Columns represent the mean value$\pm$s.e.mean of 4–6 experiments. CTR, rats not exposed to HBO for 3 h; HBO, rats exposed to hyperbaric oxygen for 3 h. Significant differences between FLUNIS-treated and untreated rats in both control and HBO-treated groups are demonstrated as $*P<0.05$ according to one-way ANOVA.

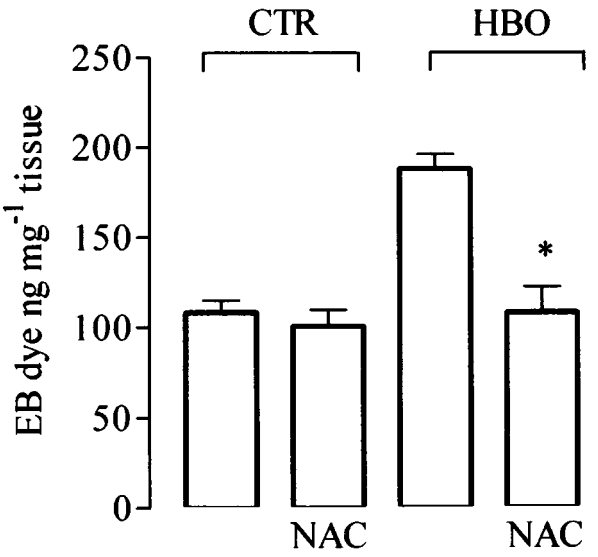

Figure 4 Effect of N-acetylcysteine (NAC) on plasma leakage of tracheal tissue in control and HBO-treated rats. Rats were treated with NAC (1 g kg^{-1} p.o.) once a day for 2 days and immediately prior to exposure to HBO or ambient air for 3 h. Columns represent the mean value$\pm$s.e.mean of 4–6 experiments. CTR, rats not exposed to HBO for 3 h. HBO, rats exposed to hyperbaric oxygen for 3 h. Significant differences between NAC-treated and untreated rats in both control and HBO-treated groups are demonstrated as $^*P<0.05$ according to one-way ANOVA.

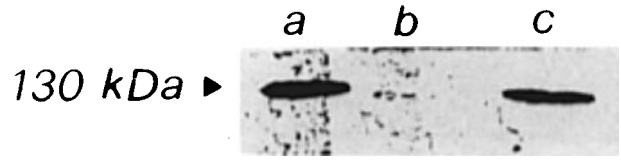

Figure 5 Western immunoblot analysis of iNOS protein in tracheal tissue of control and HBO-treated rats. Expression of iNOS protein from cytosolic fraction of tracheal homogenates of rats exposed for 3 h to HBO or ambient air (control) was investigated by Western blot analysis. The iNOS antibody recognized a protein at a molecular weight of 130 kDa. The immunoblot presents mouse macrophage cells stimulated with IFNγ (10 ng ml^{-1}) and LPS (1 μg ml^{-}) for 12 h (positive control; lane a), control (lane b) and HBO-treated (lane c). This immunoblot is representative of six separate experiments.

inhibition causes plasma leakage suggests a possible role for NO in the maintenance of microvascular integrity. In fact, different studies have suggested a protective role in the airways for constitutively formed NO (Laszlo *et al*., 1995a). L-NAME, for instance, increases plasma exudation into the trachea of vehicle for LPS-treated rats (Bernareggi *et al*., 1997). Furthermore, it has already been suggested that the maintenance of intestinal microvascular and mucosal integrity depends on the beneficial actions of NO (Laszlo *et al*., 1995b). On the contrary, as a consequence of HBO exposure, an inducible NO synthase generate a greater amount of NO which in turn is responsible for the increased tracheal vascular permeability. This hypothesis is supported by at least two observations from the present study: (1) the Western immunoanalysis of tracheal homogenates from rats exposed to HBO resulted in a significant increase in inducible NO synthase protein expression; (2) treatment of these animals with flunisolide caused a complete inhibition of the increased plasma extravasation, which is consistent with a well recognized primary mode of action of the glucocorticoids (suppression of inducible NO synthase expression) (Radomsky *et al*., 1990).

The protective effect of NAC in limiting the HBO-induced plasma leakage in rat trachea associated to the anti-exudative activity shown by L-NAME in the same group of animals suggests not also that oxygen free radicals and NO contribute largely to the injury of the large airways but that they are both related to each other. In fact, Adcock *et al*. (1994) have already demonstrated that oxidative stress induce iNOS in pulmonary alveolar epithelial cells. Even though we did not measure the expression of iNOS protein by immunoblot analysis with antibodies against iNOS in NAC-treated rats, and this should be performed in the future experiments, still NAC has been proven to significantly inhibit NO production, iNOS activity and iNOS gene expression in rat peritoneal macrophages stimulated by endotoxin (Pahan *et al*., 1998). This inhibitory effect was evident as early as 2 h pre-treatment and decreased progressively with the increase in time interval. In our study, NAC was given chronically once a day for 2 days and immediately before HBO exposure. Therefore, we could postulate that NAC would have decreased iNOS expression in tracheal homogenates of HBO-treated rats.

In our study, we were expecting that the rats exposed to HBO could demonstrate an increase in FENO, but this was not the case. The basal level of FENO in control rats was not different from that recorded in HBO exposed animals. These negative results are difficult to explain. However, we could speculate that the absence of NO in exhaled air depends on the redox, pH and biochemical milieu in the mucosa and the lining fluid of the respiratory tract resulting in other nitrogen-oxygen species than gaseous NO (Gaston *et al*., 1994; Cross *et al*., 1994a, b). Other possible interpretations might be the effects of the close relation to oxygenated haemoglobin in the lungs or the presence of radicals in the lining fluid of the respiratory tract that act as rapid scavengers for the NO formed. In fact, Schedin *et al*. (1997) were unable to obtain accumulation of NO during nose occlusion in the pig, perhaps due to local inactivation of NO by e.g. superoxide or haemoglobin. However, further experiments are needed in order to investigate if chronic exposure to HBO could increase the levels of FENO. The fact that we were able to show, indirectly, the involvement of NO in the maintenance of a normal vascular permeability does not necessarily mean that there should exist a link between exhaled NO and systemic NO production. In fact, Dillon *et al*. (1996) have already demonstrated the absence of a correlation between oral NO levels and plasma and urine nitrate and nitrite concentrations in humans suggesting that breath NO reflects local rather systemic NO production.

In this study we have found that indomethacin, a non-specific cyclo-oxygenase-1 and -2 inhibitor (Mitchell *et al*., 1993), was devoid of any inhibitory activity on the HBO-induced increase in plasma leakage in rat trachea. In agreement with our data, Mialon & Barthelemy (1991) have shown that eicosanoids do not play a major role in HBO seizures in rats. This is in contrast with another study which demonstrated that indomethacin completely eliminated the pulmonary hypertension and oedema induced by exposing rabbits for 1 h to 100% oxygen at 4 a.t.m. barometric pressure (Jacobson *et al*., 1992). Alternatively, the results presented here suggest that cyclo-oxygenase products do not play a direct role in the HBO-induced plasma leakage in tracheal tissue of rats and the major role is imputable to oxygen free radicals and NO. However, it is now recognized that cyclo-oxygenase, the first enzyme in the pathway of prostaglandin and thromboxane A_2 biosynthesis from arachidonic acid, exists in both

constitutive (COX-1) and inducible (COX-2) isoforms (Mitchell *et al*., 1995). Therefore, the development of further experiments with COX-2 selective inhibitors should be performed.

In summary, we have shown that acute exposure to HBO induces increases in rat tracheal plasma leakage. The beneficial action of NAC underlines that reactive oxygen species participate in the microvascular permeability changes observed in rat tracheal tissue. Furthermore, the inhibitory action of NOS inhibition in the HBO-induced vascular damage supports the idea that the increased production of NO, as a consequence of iNOS expression, leads to the deleterious effects observed. However, the very recent development of a new selective iNOS inhibitor, 1400W (Garvey *et al*., 1997), offers a new important tool to better characterize the role of NO when induced by HBO exposure in further experiments.

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