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The role of nitric oxide and metalloproteinases in the pathogenesis of hyperoxia-induced lung injury in newborn rats

[1]Anna Radomski, [1]Grzegorz Sawicki, [2,3,4]David M. Olson & [1,3,5,6]Marek W. Radomski

Departments of [1]Pharmacology, [2]Physiology, [3]Obstetrics and Gynaecology and [4]Perinatal Research Centre, University of Alberta, Edmonton, AB, T6G 2H7, Canada; [5]Division of Research, Lacer SA, 08025 Barcelona, Spain

1 The effects of nitric oxide (NO) and metalloproteinases (MMP-2 and MMP-9) in the pathogenesis of hyperoxia-induced lung damage in newborn rats were examined.

2 Three-day-old rat pups were subjected to hyperoxia ($\geqslant$95% O_2) or room air for 7 and 14 days.

3 Some animals were treated with N^G-L-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}, s.c., daily).

4 Histology, morphometry, oedema, Ca^{2+}-dependent and -independent NO synthase (NOS) activities, expression of NOS isoforms and the activities of MMP-2 and MMP-9 were measured in lungs of hyperoxic and control animals.

5 Exposure of rats to hyperoxia for 7 days resulted in alveolar sac injury characterized by the presence of cellular debris, red cell extravasation and inflammatory infiltration with mononuclear cells. Lung water content, epithelial, smooth muscle layers and total airway thickness was similar to controls.

6 In contrast, exposure of rats to hyperoxia for 14 days resulted in lung oedema, inflammation and epithelial proliferation.

7 Hyperoxia caused a decrease in Ca^{2+}-dependent NOS activity, an effect that was associated with increased expression of eNOS protein.

8 In control rats, Ca^{2+}-dependent NOS activity and expression of eNOS were reduced at 14 days.

9 Hyperoxia caused 10 fold increase in the activity of Ca^{2+}-independent NOS that remained significantly elevated after 14 days of exposure to hyperoxia. The activity of this enzyme was unchanged in control rats.

10 In lungs of hyperoxic rats, the immunoblot showed time-dependent, biphasic expression (peak at 7 days) of iNOS. The profile of expression of iNOS in control rats was similar.

11 The activities of MMPs were increased in lungs of hyperoxic animals.

12 The L-NAME treatment of hyperoxic animals reduced lung oedema and epithelial proliferation, but enhanced the activities of MMPs. L-NAME exerted no significant effects in control rats.

13 It is concluded that increased generation of NO contributes to the pathogenesis of hyperoxia-induced lung damage in newborn rats.

Keywords: Nitric oxide; metalloproteinase; neonatal lungs; hyperoxia

Introduction

The ventilator and oxygen therapy for neonatal respiratory distress syndrome are often complicated by hyperoxia-induced lung injury (Northway *et al*., 1967). The introduction of new therapies may have altered the severity of hyperoxia, however, its complications such as bronchopulmonary dysplasia still cause high morbidity and mortality (Farrel & Fiascone, 1997).

The pathology arises from the inflammatory response of neonatal lungs to the injury (Northway *et al*., 1967; Farrel & Fiascone, 1997). The response is characterized by the epithelial and endothelial lesion leading to an increase in vascular permeability and oedema. Chronic inflammation results in bronchial epithelial hyperplasia, airway smooth muscle proliferation, fibrosis and thickening of alveolar septa. In addition, the inflammatory processes in the lung vasculature cause intimal proliferation, smooth muscle hypertrophy and adventitial thickening.

Despite extensive research, the pathogenesis of hyperoxia-induced lung injury remains an enigma. Lipid mediators including platelet-activating factor, thromboxane and leukotrienes have been all implicated in the disease process (Northway *et al*., 1967; Farrel & Fiascone, 1997; Boros *et al*., 1997). However, pharmacological inhibition of lipid mediators did not attenuate lung inflammation. Indeed, inhibition of leukotriene formation by a specific 5-lipoxygenase-inhibitor/receptor antagonist Wy-50295 exerted no significant effect on lung water content (Boros *et al*., 1997). It is now clear that the pro-inflammatory cytokines play central role in the pathophysiology of inflammation (Dinarello & Thompson, 1991). Exposure of lungs to cytokines results in expression of inducible enzymes including nitric oxide synthase (NOS) and metalloproteinases (MMPs).

Nitric oxide synthase is the family of isoformic enzymes converting L-arginine to NO and L-citrulline (Radomski, 1995). Gene cloning has identified at least three isoforms of NOS including the endothelial type (eNOS), neuronal (nNOS) and the isoform expressed during inflammation by cytokines, iNOS. The eNOS is Ca^{2+}-dependent, expressed in the endothelial cells and platelets, and generation of physiological amounts of NO by this enzyme plays an important role in regulation of vascular reactivity, platelet and leukocyte functions. In contrast, iNOS is Ca^{2+}-independent, generates large amounts of NO, that either *per se*, or following

[6]Author for correspondence at: Department of Pharmacology, University of Alberta, Edmonton, AB, T6G 2H7, Canada.

conversion to secondary oxidants such as peroxynitrite ($ONOO^-$) (Radomski & Salas, 1995; Beckman *et al*., 1990), contribute to the pathogenesis of lung injury.

An excessive remodelling associated with inflammation is caused by metalloproteinases (MMPs), matrix-degrading enzymes such as MMP-2 and MMP-9. These enzymes are synthesized as latent forms (pro-MMP-2 and pro-MMP-9) that are subsequently activated by limited proteolysis to MMP-2 and MMP-9 (Goetzl *et al*., 1996). Increased expression of MMPs has been associated with lung damage (Shapiro, 1994).

We, therefore, examined the generation of NO and MMPs during the inflammatory reactions in newborn rats exposed to normobaric hyperoxia. We hypothesized that both iNOS and MMPs were up regulated by hyperoxia and contributed to the pathogenesis of lung injury in newborn rats.

Methods

Animals

Sprague-Dawley rat pups (Charles River Laboratories, Wilmington, MA, U.S.A.) of both sexes were used. Dams were maintained on regular rodent diet, water *ad libitum* and kept on a 12-h light/dark cycle. The animals were housed under veterinary supervision.

The guidelines of the Canadian Council of Animal Care were followed in all experimental procedures.

Treatments

Parallel litters (24 litters total 288 pups) of randomly chosen 3-day-old rat pups were exposed to hyperoxia for 7 and 14 days as described before (Boros *et al*., 1997). Briefly, pups were kept in exposure chambers containing $\geqslant 95\%$ oxygen or room air. Carbon dioxide levels, temperature and humidity were maintained at $<0.5\%$, 26°C and 75–80%, respectively. Chambers were opened for 15 min daily, to switch dams between oxygen and air environments.

Some animals were treated with either N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1} s.c., per day), an isoform-non-selective NOS inhibitor (Rees *et al*., 1990) or placebo (physiological saline). In the preliminary experiments it was found that this dose of L-NAME, when administered for 14 days during exposure of rats to hyperoxia for the same period of time, reduced Ca^{2+}-dependent and -independent NOS activities in lungs by 52 ± 6 and $67 \pm 12\%$, $n=5$, $P<0.05$, respectively. Higher doses resulted in complete inhibition of NOS activity in lungs. The activity of NOS was measured as the rate of conversion of ^{14}C-L-arginine to ^{14}C-L-citrulline as described before (Radomski *et al*., 1993, see below).

At the end of experiments rats were euthanized using an overdose of sodium pentobarbitone (100 mg kg^{-1}, Euthanyl TM MTC Pharmaceuticals, Cambridge, ON, U.S.A.). The pups were exsanguinated and organs perfused through the right ventricle with 5 ml physiological saline. Lungs were then removed, frozen in liquid nitrogen and stored until assayed at −80°C.

In some experiments tissue specimens were fixed for morphometric analysis. Lungs were fixed *in situ via* a tracheal canula with 10% phosphate-buffered formalin (Sigma Chemical Co., Mississauga, ON, Canada) at a constant pressure of 20 cm H_2O for 2 h. Then, the trachea was ligated, lungs excised and immersed in formalin for up to 48 h.

Airway histology and morphometry

Following fixation, transverse sections of superior, middle and inferior lobes of right lung and superior and inferior sections of left lung were embedded in paraffin. The sections were stained with Gomori-trichrom.

Light microscopy morphometric evaluation of airway layer fractional areas was carried out using an image analysis system consisting of a microscope (Carl Zeiss Variant Jenamed), video camera (MITS 68), computer (386 Modular PC, IBM), and image-analysis software (Genias 25, Joyce-Loebl Co.). Airways cut obliquely (as defined by circularity $4\pi \times \text{area}\ \text{perimeter}^{-2}$, <0.7 or >1.3) were eliminated from analysis. The resulting number of circularly cut small airways was on average 8–10 per animal. Only airways with a circumference less than 500 μm, as defined by the total airway wall perimeter, were measured. The morphometry analysed the area of the total airway wall including the epithelial layer and the smooth muscle layer. Each measurement was normalized in relation to the basement membrane circumference (Boros *et al*., 1997).

Extravascular lung water (lung oedema)

The extravascular lung water content was measured as a difference between wet and dry lung weights. Briefly, the rats were euthanized, and exsanguinated. The lungs were then removed from the chest cavity, minimally blotted using cotton swab and wet weights determined. The lungs were allowed to dry in the oven at 37°C and were weighed daily. The tissue was considered dry when the weight was constant for 2 consecutive days (usually 3 days later), and dry weight was recorded.

Lung samples

Frozen samples of lungs were crushed on dry ice and then sonicated using a Vibra Cell sonicator (Sonics and Materials Inc., Danbury, CT, U.S.A.) in 400 μl of ice-cold homogenizing buffer (Radomski *et al*., 1993) for 10 s at 4°C. The homogenates were centrifuged for 20 min at 10,000 g at 4°C and the supernatant used as a source of NOS and MMPs.

Nitric oxide synthase

The activity of NOS was measured as the rate of conversion of ^{14}C-L-arginine to ^{14}C-L-citrulline as described before (Radomski *et al*., 1993). Briefly, the samples were incubated at 37°C with ^{14}C-L-arginine (Amersham) in assay buffer containing 50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM L-Valine, 1 mM L-citrulline, 20 μM L-arginine, 0.1 mM NADPH, 10 μM tetrahydrobiopterin and 1.5 mM dithiothreitol, in the presence or absence of 1.5 mM N^G-monomethyl-L-arginine (L-NMMA). EGTA (2 mM) was used to differentiate between Ca^{2+}-dependent and Ca^{2+}-independent NOS (Radomski *et al*., 1993). After 20 min incubation the reaction was terminated by dilution and removal of non-reacted L-arginine using AG 50W-X8 resin (Bio-Rad) and the remaining radioactivity counted using a liquid-scintillation counter (Beckman).

Expression of NOS isoforms was studied using Western blot analysis. Samples (80 μg protein each) were subjected to 7% SDS–PAGE under reducing conditions (Laemmli, 1970). Proteins were transferred onto polyvinylidene fluoride membranes (Schlecher & Schuell, Keene, NH, U.S.A.) using a Trans-Blot Cell system (Bio-Rad, Mississauga, ON, U.S.A.). The eNOS, nNOS and iNOS isoforms were identified using respective polyclonal antibodies (Santa Cruz, La Jolla, CA, U.S.A., 0.1 μg ml^{-1}). Blots were developed using an ECL kit

(Amersham) and the density of bands was quantified using a ScanJet 3c scanner (Hewlett Packard, Boise, ID, U.S.A.) and SigmaGel measurement software (Jandel Corporation, San Rafael, CA, U.S.A.).

Zymography

The activities of MMP-2 and MMP-9 were measured by zymography under non-reducing conditions as described before (Sawicki *et al*., 1997, 1998). Briefly, samples (20 μg protein each) were subjected to 8% SDS–PAGE with copolymerized gelatine (2 mg ml^{-1}) as a substrate. The gelatinolytic activities of MMP-2 and MMP-9 were read and quantified by measuring the density of the bands using a ScanJet 3c scanner and SigmaGel measurement software.

Statistics

Results are expressed as the mean $\pm$ s.e.mean and comparisons were made using one-way analysis of variance followed by Tukey-Kramer multiple comparison test. A probability $P < 0.05$ was taken as statistically significant.

Reagents

Unless otherwise indicated all reagents were purchased from Sigma. N^G-monomethyl-L-arginine was from Glaxo-Wellcome.

Results

Animals

Hyperoxia resulted in a small (approximately 10%) reduction in the weight of rat pups (Table 1). Two out of 144 exposed pups died during exposure to hyperoxia.

Airway histology and morphometry

In the lungs of rats exposed to hyperoxia hyperaemia was detected after 7 days (Figure 1A). In alveolar sacs cellular debris, extravasation of red cells and the infiltrate composed mainly of mononuclear cells were also detected (Figure 1B). The airways of control animals were lined with one layer of ciliated, cuboidal epithelial cells (Figure 1C) with normal parenchyma (Figure 1D).

Exposure of animals to hyperoxia for 14 days resulted in the epithelial hyperplasia. The airways were lined with several layers of epithelial cells forming polypoid-like structures that were protruding into the lumen of the airways (Figure 2A). In the airway walls and within the alveoli some inflammatory infiltrate composed mainly of lung histiocytes was also detected (Figure 2B). L-NAME reduced the pathological changes caused by hyperoxia (Figure 2C and D). The airways of control animals appeared normal upon histological inspection (Figure 2E and F).

In rats exposed to hyperoxia for 7 days, the morphometric analysis of the airway epithelium and smooth muscle cells was similar to controls (data not shown). In contrast, exposure of rats to hyperoxia for 14 days caused a significant epithelial proliferation in the airways, an effect that was significantly attenuated by L-NAME (Figure 3A). The airway smooth muscle layer and total airway wall thickness were not altered significantly by hyperoxia (Figure 3B and C). The airway epithelium and smooth muscle of control animals were not affected by L-NAME treatment (Figure 3).

Lung oedema

The rats subjected to hyperoxia for 14 days showed a significant increase in water content in the lungs consistent with induction of pulmonary oedema (Figure 4). This effect

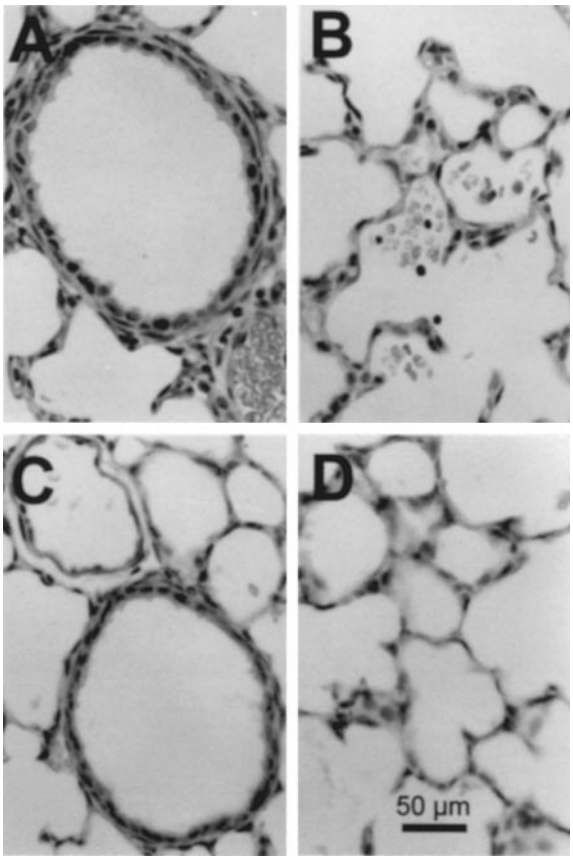

Figure 1 Light microscopy of airways of newborn rats exposed to room air or hyperoxia for 7 days. (A) Transverse section of lung airway of a hyperoxic rat pup. Note the presence of hyperaemic vessel associated with the airway. (B) The section shows cellular debris, extravasated red cells and mononuclear inflammatory infiltrate within an alveolar sac of hyperoxic rat. (C) Transverse section of control lung airway shows one layer of cuboidal, cilliated epithelial cells. (D) Lung parenchyma of control rat pup.

Table 1 Hyperoxia treatment and weights of rat pups

Time (weeks)	*Hyperoxia* (g)	*Control* (g)	*Hyperoxia* + L-*NAME* (g)	*Control* + L-*NAME* (g)
Before treatment	7.8 $\pm$ 0.8	7.9 $\pm$ 0.4	8.4 $\pm$ 0.7	8.3 $\pm$ 0.6
One week	17.2 $\pm$ 1.9	18.3 $\pm$ 1.7	18.6 $\pm$ 1.5	19.9 $\pm$ 1.6
Two weeks	26.0 $\pm$ 3.8*	30.1 $\pm$ 0.8	25.3 $\pm$ 2.3**	29.6 $\pm$ 2.1

Data are mean $\pm$ s.e.mean (g), $n = 8-9$ per group. *$P = 0.0106$. **$P = 0.0201$ treatments *vs* respective controls.

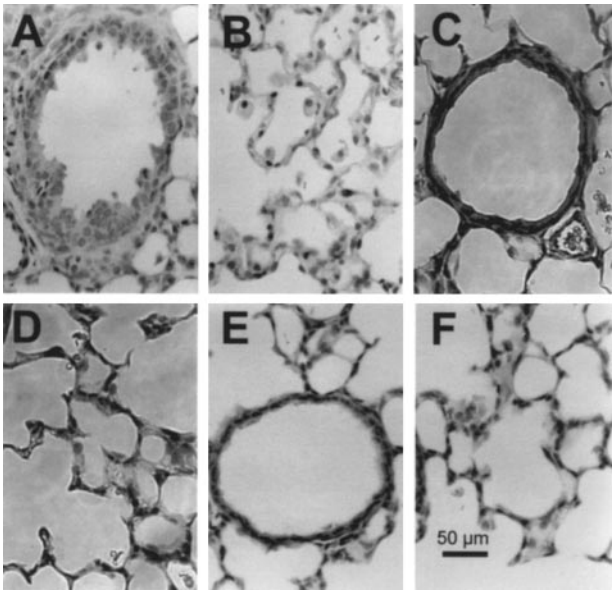

Figure 2 Light microscopy of airways of newborn rats exposed to hyperoxia for 14 days. (A) Section of airway of a hyperoxic rat pup shows several layers of hyperplastic epithelium. (B) The histiocytic infiltrate of lung parenchyma of hyperoxic rat. (C) Section of airway of a hyperoxic rat treated with L-NAME shows a decrease in epithelial proliferation and histiocytic infiltration (D). (E) and (F) Histological appearance of lung airway and parenchyma of control animals.

of hyperoxia was significantly attenuated by the treatment with L-NAME. The treatment of control animals with L-NAME exerted no significant effect on lung water content (Figure 4).

Nitric oxide synthase

Exposure of rats to hyperoxia for 14 days caused a decrease in Ca^{2+}-dependent NOS activity in the lungs, an effect also detected in control animals (Figure 5A). Furthermore, there was a significant reduction of Ca^{2+}-dependent NOS in the lungs of hyperoxic animals when compared to controls following 14 days of exposure to oxygen (Figure 5A). Western blot analysis showed a significant reduction of eNOS expression with age in control rat pups (Figure 5B and C). In pups subjected to hyperoxia there was a significant increase in expression of eNOS after 7 days of exposure, an effect not detectable after 14 days of exposure (Figure 5B and C).

The Ca^{2+}-independent NOS activity in the lungs was increased by hyperoxia such that it was maximal after 7 days of exposure (Figure 6A). Although by day 14 this activity decreased, it was still significantly elevated compared to controls (Figure 6A). In control rats, the activity of this enzyme remained low and unchanged over the experimental period. Western blot analysis showed bell-shaped increase in expression of iNOS in the lungs of hyperoxic and control rats that was maximal at 7 days (Figure 6B and C).

Expression of nNOS was not detected in the lungs of control and hyperoxic rats (data not shown).

Zymography

The lungs of normoxic rats expressed pro-MMP-9, pro-MMP-2 and MMP-2. Exposure of animals to hyperoxia for 14 days resulted in a significant increase in the activity of these enzymes, an effect significantly potentiated by treatment

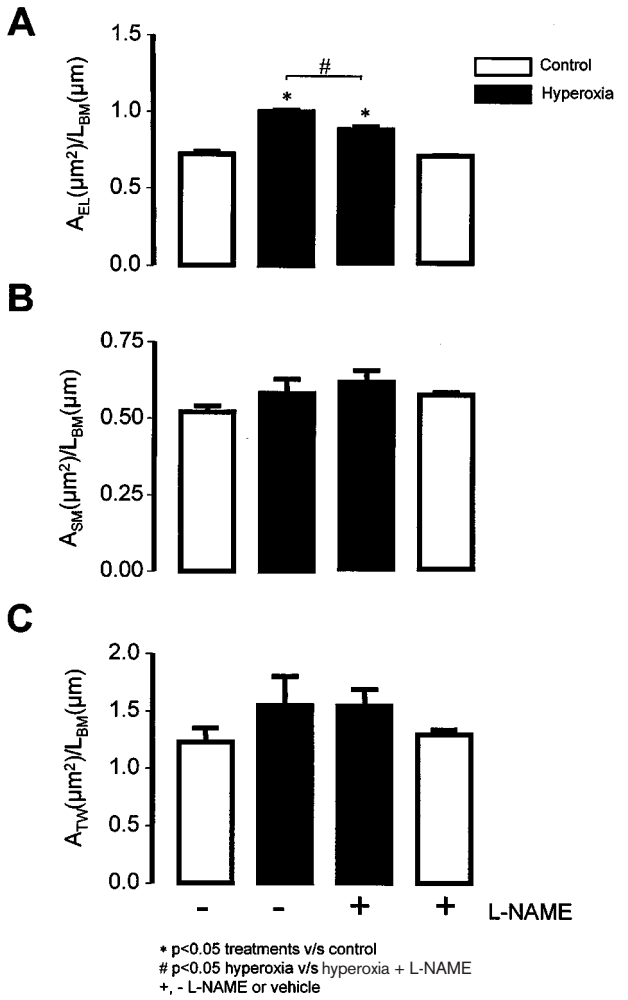

Figure 3 Airway morphometry of rats subjected to hyperoxia for 14 days. Hyperoxia resulted in epithelial proliferation, an effect attenuated by L-NAME (A). Smooth muscle layer (B) and total airway wall area (C) were not significantly altered by hyperoxia. L-NAME exerted no significant effects on airways of control animals (A), (B) and (C). $n = 5$ pups per group.

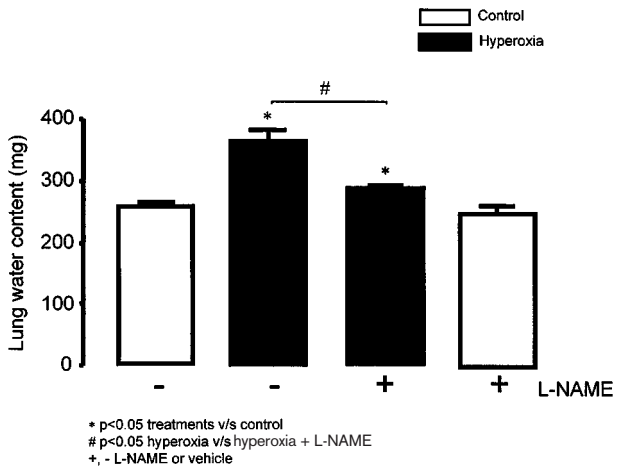

Figure 4 The effect of hyperoxia on lung water content in newborn rats. Hyperoxia resulted in increased water content in lungs of rat pups, an effect attenuated by L-NAME. The inhibitor exerted no significant effect on lung water content of control animals. $n = 7-16$ rats per group.

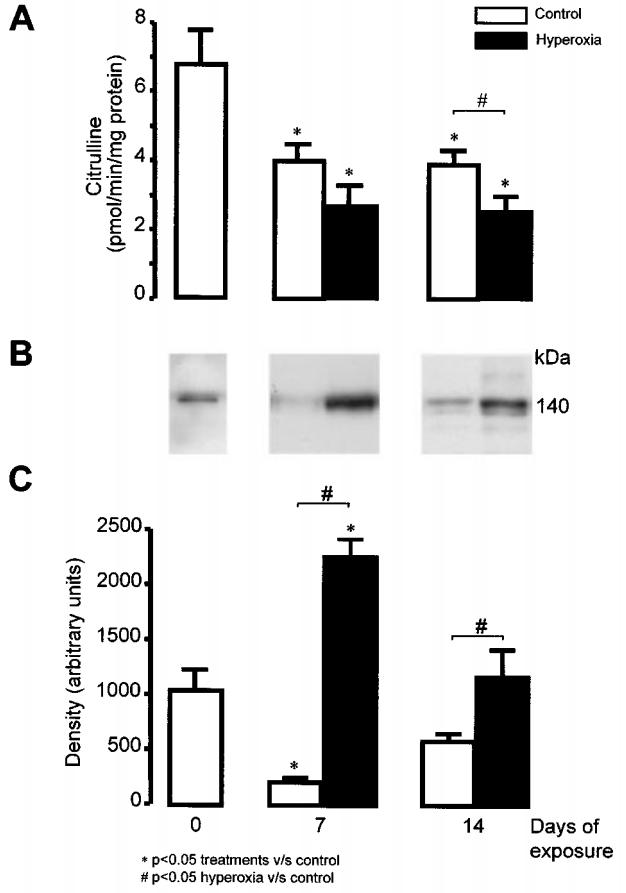

Figure 5 The changes in the activity of Ca^{2+}-dependent NOS and expression of eNOS immunoreactivity caused by hyperoxia. (A) The activity of Ca^{2+}-dependent NOS was significantly decreased in control and hyperoxic rats with age. There was a further reduction of the activity of this enzyme in lungs of rats exposed to hyperoxia for 14 days. (B) and (C) The immunoblots showing the changes in expression of eNOS in lungs of hyperoxic and normoxic rats. $n=8$ rats per group.

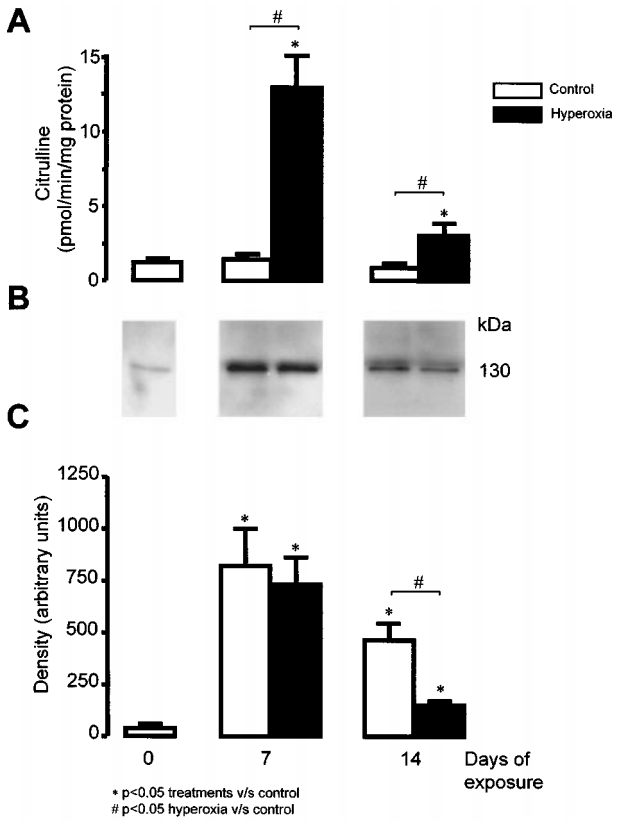

Figure 6 The changes in the activity of Ca^{2+}-independent NOS and expression of iNOS immunoreactivity caused by hyperoxia. (A) Increased activity of Ca^{2+}-independent NOS in hyperoxic rats. The activity of enzyme was maximal after 7 days of exposure and was still elevated after 14 days compared to controls. (B) and (C) The immunoblots show the changes in expression of iNOS in lungs of hyperoxic and normoxic rats. $n=8$ pups per group.

with L-NAME (Figure 7A and B). L-NAME exerted no significant effect on the activity of MMPs in normoxic rats (Figure 7A and B).

Discussion

We have shown that exposure of newborn rats to hyperoxia for 14 days, but not 7 days, resulted in lung damage characterized by pulmonary oedema, epithelial proliferation and inflammatory infiltration. In these characteristics, hyperoxia-induced lung damage in rat pups resembles bronchopulmonary dysplasia (Northway *et al*., 1967; Farrel & Fiascone, 1997), however, it differs from this human pathology because of the lack of appreciable bronchial smooth muscle proliferation. The development of lesion was associated with down-regulation of Ca^{2+}-dependent NOS activity and up-regulation of Ca^{2+}-independent NOS activity in the lungs of newborn animals.

Nitric oxide is a major pulmonary vasodilator (Crawley *et al*., 1990; Dinh-Xuan, 1992) bronchodilator (Nijkamp & Folkerts, 1997) and regulator of platelet haemostasis (Radomski & Salas, 1995). A reduction of Ca^{2+}-dependent NOS activity in neonatal lungs due to hyperoxia observed in our experiments, as well as in the hyperoxic lung of adolescent/adult mice (Arkovitz *et al*., 1997) may facilitate vasoconstriction, bronchoconstriction and platelet and leukocyte activation.

A 10 fold increase in the activity of Ca^{2+}-independent NOS in rat pups, measured at day 7 of hyperoxia, preceded the development of morphological lesion that became detectable following 14 days of exposure to 95% oxygen. Interestingly, exposure of adolescent/adult mice to hyperoxia for up to 5 days did not result in increased expression of iNOS (Arkovitz *et al*, 1997). It is likely that both sensitivity of neonatal v/s adult lungs to hyperoxia and duration of exposure account for this difference.

Cytokine-induced expression of this enzyme, generation of large amounts of inducible NO, and possibly peroxynitrite, have been associated with acute and chronic lung damage including septicemia, ischemia-reperfusion injury and bronchial asthma (Barnes & Belvisi, 1993; Kooy *et al*., 1995; Ischiropoulos *et al*., 1995). Interestingly, elevated levels of pro-inflammatory cytokines were detected in patients with BPD (Tullus *et al*., 1996, Rindfleisch *et al*., 1996). Moreover, L-arginine, the substrate for the synthesis of NO, enhanced hyperoxia-induced rabbit lung injury through a mechanism that could involve generation of peroxynitrite (Nozik *et al*., 1995). Thus, stimulation of NOS is likely to contribute to the pathogenesis of lung injury induced by hyperoxia.

We have detected the changes in expression of NOS in the lungs of pups subjected to hyperoxia. It has been recently shown (Gess *et al*., 1997) that acute hypoxia in rats results in up-regulation of mRNAs for eNOS in the lungs. Interestingly, the intrapulmonary artery endothelial cells in culture, when

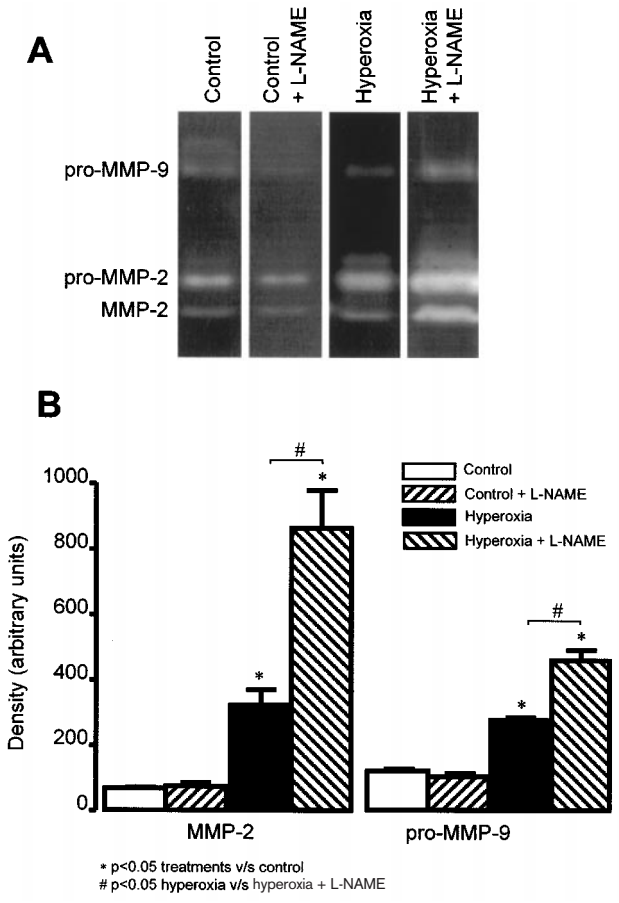

Figure 7 The effects of exposure to hyperoxia for 14 days on the activities of MMP-9 and MMP-2 in lungs of newborn rats. The activities of pro-MMP-9 (100 kD), pro-MMP-2 (72 kD) and MMP-2 (64 kD) in control and hyperoxic rats. Representative zymograms are shown. Densitometric analysis of zymograms show potentiation of hyperoxia-induced increases in pro-MMP-9 and MMP-2 activities by L-NAME. L-NAME exerted no significant effect on MMPs activity in normoxic rat pups.

exposed to hyperoxia, showed up-regulation of eNOS protein that was associated with increased enzyme activity (North *et al*., 1996). In our experiments with newborn rat pups hyperoxia caused a transient up-regulation of expression of eNOS protein that was associated with a decrease in the activity of this enzyme. These data suggest that, both hypoxia and hyperoxia affect the activity and expression of eNOS. We have also detected increased expression of iNOS in the lungs of pups subjected to hyperoxia, however, this expression was similar to that in control animals.

It is very important to note that the changes in expression and activity of NOS induced by hyperoxia take place against the background of alterations brought about by ageing and maturation of rat pups. It has been previously shown that expression of three isoforms of NOS is present and regulated during early lung development (Xue *et al*., 1996). Here, we showed that both eNOS and iNOS are modulated in the post-natal period in rats. There was a good correlation between the changes in expression and activity of eNOS, as both Ca^{2+}-dependent citrulline formation and the immunoreactive protein decreased over the period of experiment. Interestingly, there was a dramatic up-regulation of expression of iNOS in control rats over the period of 7 days. It is tempting to speculate that exposure of newborn pups to non-sterile environment different from that in the foetal life is responsible for this transformation.

The growth and maturation of neonatal lungs are dependent on remodelling of the lung structure and MMP-2 appears to play a major role in this process (Arden & Adamson, 1992; Arden *et al*., 1993). However, an excessive activation of matrix-remodelling enzymes is clearly detrimental as it leads to the destruction of lung architecture (Devaskar *et al*., 1994). We have found that hyperoxia increased the activities of lung MMPs in newborn rats. This is in agreement with previous work that demonstrated increased expression of type IV collagenase cDNA and mRNA following exposure of adult rats to hyperoxia (Devaskar *et al*., 1994). Thus, increased activity of MMPs caused by hyperoxia is likely to contribute to the degradation of the extracellular matrix and lung damage observed in hyperoxia-induced lung injury.

To investigate the pathological significance of hyperoxia-induced changes in NOS activity for the pathogenesis of lung damage we treated rats with L-NAME (Rees *et al*., 1990). We selected 10 mg kg^{-1} s.c. per day L-NAME that in preliminary experiments reduced total NOS activity in the lungs by approximately 60%. The rationale for the choice of this dose of L-NAME was to minimize its impact on vasoconstriction and blood cell activation that might have resulted from a non-selective inhibition of the constitutive, Ca^{2+}-dependent eNOS (Laszlo *et al*., 1994).

We found that the treatment with L-NAME of newborn rats reduced lung oedema. This is similar to the experiments with adult rats exposed to hyperoxia that showed that inhibition of NOS decreased water content in lungs (Capellier *et al*., 1996). The treatment of rat pups with L-NAME also led to reduction of hyperoxia-induced epithelial proliferation. Interestingly, in the epithelium of patients suffering from idiopathic pulmonary fibrosis, a disease characterized by alveolar inflammation, progressive proliferation of septal cells and loss of lung architecture, there is evidence for expression of iNOS and generation of peroxynitrite (Saleh *et al*., 1997). Peroxynitrite is a strong oxidant derived from NO and superoxide (Beckman *et al*., 1990) and its generation may contribute to the oxidative lung injury during ischaemia-reperfusion (Ischiropoulos *et al*., 1995). Both stimulation of constitutive (Ischiropoulos *et al*., 1995) and expression of inducible NOS (Saleh *et al*., 1997) may result in generation of $ONOO^-$. As L-NAME inhibits all isoforms of NOS (Rees *et al*., 1990), the pharmacological effects of this inhibitor on oedema and epithelial proliferation may stem from non-selective inhibition of NO generation in hyperoxic lungs. It is, however, relevant to point out that in our model hyperoxia led to stimulation of Ca^{2+}-independent iNOS, but not Ca^{2+}-dependent eNOS activity.

We also found that L-NAME greatly potentiated the activities of lung MMPs that had been already elevated as a result of hyperoxia without affecting the gelatinolytic activity in the lungs of normoxic animals. In platelets, MMP-2 is translocated to the platelet surface membrane and then released upon cell activation (Sawicki *et al*., 1997, 1998). As NO inhibits this release (Sawicki *et al*., 1997), inhibition of NOS in the hyperoxic lungs by L-NAME could disrupt the NO-mediated regulatory mechanism controlling MMPs secretion. Interestingly, inhibition of NOS with L-NAME failed to enhance the activity of MMPs in the lungs of normoxic rat pups. This indicates that in neonatal lungs, under basal conditions, factors other than NO regulate the release and activity of MMPs. These may include gelatinase associated lipocalin (Kjeldsen *et al*., 1993) and tissue inhibitors of metalloproteinases (Goetzl *et al*., 1996). It has been also

shown that reactive oxygen species produced by macrophage-derived foam cells stimulate the activity of MMPs (Rajagopalan *et al*., 1996). In addition, in cultured rabbit articular chondrocytes stimulated with interleukin-1, inhibition of NOS reduced both expression and activity of MMPs (Tamura *et al*., 1996; Sasaki *et al*., 1998). In contrast, cytokine inducible MMPs expression in immortalized rat chondrocytes was not modified by NOS inhibition (Horton *et al*., 1998). The reasons for such diverse effects of NO on MMPs are not clear, however, a differential regulation of MMPs expression, release and activation *in vitro* vs *in vivo* may account for this discrepancy.

Under some experimental conditions, L-NAME has been reported to increase mortality of adult rats and mice subjected to hyperoxia (Arkovitz *et al*., 1997; Garat *et al*., 1997). It is important to indicate that adult rats are more sensitive to hyperoxia than pups, as in our experiments the mortality of newborn animals exposed to high concentrations of oxygen was very low. Moreover, in the experiments that showed detrimental effects of L-NAME on animal survival during hyperoxia, this NOS inhibitor was administered at high doses (ranging from 60 to 100 mg kg^{-1} day^{-1} i.p., and 50 mg kg^{-1} day^{-1} p.o.) likely to abolish the activity of eNOS and precipitate vascular toxicity.

Thus, the net pharmacological effect (beneficial or detrimental) of L-NAME on hyperoxia-induced lung damage appears to be dose-dependent. Partial inhibition of increased NO formation by the inhibitor decreases lung water content and epithelial proliferation leading to attenuation of these two symptoms of chronic inflammation. The pharmacological significance of increased activities of MMP-2 and MMP-9 caused by L-NAME is unclear. Increased activation of MMPs is often associated with increased remodelling of the extracellular matrix and profound changes in tissue architecture (Shapiro, 1994). However, despite large enhancement of MMPs activities, L-NAME clearly decreased epithelial proliferation and lung oedema, which were increased by hyperoxia. Thus, it is possible that, in hyperoxia-induced lung damage in newborn rats, inducible NO, but not MMPs, is the major determinant of injury. Further work is needed to establish the functional significance (i.e. the significance for the respiratory function) of activation of MMPs by non-selective inhibition of NOS in lung inflammation. Recently, highly selective inhibitors of iNOS and MMPs have become available for the experimental and clinical use, respectively (Garvey *et al*., 1997; Gordon *et al*., 1993). It would be interesting to test the pharmacological effectiveness of these compounds in the setting of hyperoxia-induced lung damage.

The current neonatal intensive care management of bronchopulmonary dysplasia, that is often a complication of oxygen therapy in acutely-ill neonates (Northway *et al*., 1967; Farrel & Fiascone, 1997), includes also the treatment with inhaled NO. Inhaled NO gas has been used to correct ventilation/perfusion mismatching in critically-ill infants (Frostell & Zapol, 1995) including a group of children with BPD (Mupanemunda & Silverman, 1994). It remains to be demonstrated whether the use of NO gas affects the course of hyperoxia-induced lung damage in critically ill neonates.

In conclusion, we have shown that hyperoxia-induced lung injury is associated with increased generation of inducible NO. We suggest that these changes may play a role in the pathogenesis of hyperoxia-induced lung damage. Pharmacological relevance of our findings remains to be investigated.

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