

L-NAME aggravates pulmonary oxygen toxicity in rats

G. Capellier*, V. Maupoil**, A. Boillot⁺, J-P. Kantelip⁺⁺, L. Rochette[‡],
J. Regnard^{‡‡}, F. Barale^{**}

L-NAME aggravates pulmonary oxygen toxicity in rats. G. Capellier, V. Maupoil, A. Boillot, J-P. Kantelip, L. Rochette, J. Regnard, F. Barale. ©ERS Journals Ltd 1996.

ABSTRACT: Exposure to high oxygen concentration leads to acute lung injury and death in rats after 72 h. The pathophysiology of this phenomenon relies on several mechanisms, including alteration of vascular reactivity, recruitment and activation of neutrophils and alveolar macrophages, production of cytokines and excess production of free radicals. In addition to its potent vasodilating effect, nitric oxide (NO) has also been reported to prevent free radical-mediated damage. We wanted to determine whether N^G-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor, might modulate oxygen toxicity.

In rats exposed to continuous high oxygen concentration, we studied the effect of administration of 50 mg·kg⁻¹ of intraperitoneal L-NAME twice a day on the first day of oxygen exposure.

L-NAME resulted in earlier death, since 57% of the animals exposed to oxygen and injected with L-NAME died within 60 h as compared to 22% of the animals exposed to oxygen and treated with saline (p<0.01). Haematocrit and bronchoalveolar lavage fluid protein were also significantly increased in animals exposed to oxygen and receiving L-NAME. The lung water content was higher in the oxygen-exposed groups (p<0.01) and slightly decreased by L-NAME (p<0.05). Thiobarbituric acid reactive substances (TBARS) were elevated in plasma (p<0.01) and decreased in lung (p<0.001) of oxygen-exposed animals, but no significant effect of L-NAME was observed.

N^G-nitro-L-arginine methyl ester had a deleterious effect in rats exposed to hyperoxia, which might suggest that endogenous nitric oxide has a protective role against hyperoxia-induced pulmonary lesions.

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*Réanimation médicale, +Anesthésie-Réanimation, ++Pharmacologie fondamentale, and ‡‡Physiologie et Exploration Fonctionnelle Rénale, CHU Jean Minjoz, Besançon, France. **Physiologie et Pharmacologie-Nutrition Préventive Expérimentale, Faculté de Pharmacie, Besançon, France. ‡LPPCE, Faculté de Médecine, Dijon, France.

Correspondence: G. Capellier
Réanimation médicale
Centre Hospitalier Universitaire Jean Minjoz
Boulevard Fleming
25030 Besançon
France

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Oxygen toxicity in animals is well-described, with acute lung injury occurring within a few days in most animals species. In rats exposed to oxygen, death usually occurs in 3–4 days [1]. The pathophysiology of oxygen toxicity relies on several mechanisms, including alteration of vascular reactivity [2, 3], recruitment and activation of neutrophils and alveolar macrophages [4], production of cytokines and denaturation of deoxyribonucleic acid (DNA) [5]. However, the predominant mechanism is likely to be the excessive production of free radicals [6]. Reactive oxygen species, such as superoxide and hydroxyl radicals, are important mediators of lung injury, whether they are produced inside lung parenchyma cells or by immigrant neutrophils [7].

There is also circumstantial evidence that high oxygen concentrations stimulate both constitutive [8] and inducible [9] nitric oxide (NO) synthases and increase NO production. Alveolar macrophages increase their production of tumour necrosis factor- α (TNF- α) in rats breathing hyperoxic gas, and TNF- α potently stimulates the activity of inducible NO synthase (iNOS) [10, 11]. Similarly, another oxidant, ozone, augments the expression of iNOS and the production of reactive nitrogen compounds in alveolar type II rat cells [12].

Although NO may react with oxygen to yield highly reactive and toxic compounds, such as peroxynitrite [10, 13], NO also inactivates oxygen free radicals. NO may impede the formation of the hydroxyl radical equivalents that result from the metal-mediated reduction of hydrogen peroxide [14]. In oxygenated blood, NO may act as a chemical barrier to cytotoxic oxygen free radicals [15]. In cultured fibroblasts from hamster lungs exposed to hypoxanthine/xanthine oxidase or hydrogen peroxide, adding an NO donor markedly decreased the superoxide or hydrogen peroxide cytotoxicity [14]. In isolated rabbit lungs perfused with the xanthine-xanthine oxidase superoxide generating system, inhaled NO prevented oedema formation [16]. It is also hypothesised that NO protects against cellular damage likely to occur during reperfusion following lung ischaemia [17]. It was recently demonstrated that an NO-precursor, L-arginine [18] and inhaled NO [4] prevented ischaemia-reperfusion lung injury. In addition, the inhibition of NO synthase increased injury due to oxygen free radicals [19, 20]. Free radical-mediated hepatotoxicity induced by carbon tetrachloride and lipopolysaccharide (LPS) is enhanced by the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA), suggesting a protective role for

NO [21]. Another NO synthase inhibitor, aminoguanidine, increases tissue damage to the lungs in mice injected with LPS and formyl-norleucyl-phenylalanine (FNLP) (PHENG [22]). Inhibition of NO synthase markedly increased the hepatic damage following LPS injection in a preparation of chronic hepatic inflammation [23], and also amplified histological damage and vascular permeability in endotoxin-induced acute intestinal damage of the rat [24].

Conversely, the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) had a protective effect against lung injury induced by the deposition of immune complexes [25], and also against alveolar injury caused by smoke inhalation [26]. We, therefore, wanted to determine whether L-NAME, a NO synthase inhibitor, might modulate long-term oxygen toxicity in rats breathing in pure oxygen.

Material and methods

Oxygen exposure

Male Sprague-Dawley rats (Iff'a-Credo, Lyon, France), weighing 275–325 g, were housed in an animal facility for 1 week before entering the protocol. Oxygen exposure was performed through a continuous oxygen flow (2 L·min⁻¹) delivered in sealed plexiglass chambers (Bioblock Scientific, Illkirch, France). Inspiratory oxygen fraction (F_{I,O_2}) was maintained higher than 99%, and expiratory carbon dioxide fraction (F_{E,CO_2}) lower than 1%. These values were checked every 12 h with an Oxydig oxygen fraction (F_{O_2}) monitor (Drägerwerk, AG Lübeck, Germany), and a Capnolog carbon dioxide fraction (F_{CO_2}) monitor (Robert et Carrière, Dräger, Anthony, France). Temperature inside the chambers was maintained between 20–22°C. Relative humidity of gas entering the chambers was 70% (checked every 12 h with a 6010 Hygrometer; Testoterm, Forbach, France). Animals had direct access to food and water in the exposure chambers. Control animals were exposed to room air.

Experimental protocol

On the first day of the experiment, each rat received two intraperitoneal (*i.p.*) injections, 12 h apart, of either normal saline or 50 mg·kg⁻¹ L-NAME (Sigma Saint-Quentin-Fallavier, France). According to the gas they breathed in and the *i.p.* treatment received, four animal groups were considered: Air-Saline (A-S); Air-L-NAME (A-LN); Oxygen-Saline (O-S); and Oxygen-L-NAME (O-LN).

For the mortality study, deaths were rated every 12 h at 08:00 and 20:00 h. In the first part of the mortality study, 28 animals were randomly allocated to the four groups. The absence of mortality in the air-breathing groups led us to consider only the oxygen-breathing groups. In a second phase, oxygen-breathing rats were randomly allocated either to L-NAME or normal saline.

Lung injuries were studied in a further series of animals. After 36 h exposure, the animals were anaesthetised with *i.p.* thiopental (50 mg), and then received intravenous heparin (10 mg·kg⁻¹) before the rapid opening of their abdomen and drawing of blood samples for

haematocrit and concentration of thiobarbituric acid reactive substances (TBARS). After exsanguination by transection of the aorta and vena cava, the chest was rapidly opened. The left lung was dissected, removed, blotted dry, weighed and used for lung water measurement after desiccation at 110°C for 36 h. The trachea was then intubated with a 16-gauge catheter, and a 10 mL saline bronchoalveolar lavage (BAL) was gently performed. After centrifugation at 800 rpm for 10 min at 4°C, aliquots of the recovered BAL fluid (BALF) were prepared for determination of protein, potassium, lactic dehydrogenase (LDH) enzyme, cyclic guanosine monophosphate (cGMP) and nitrates. BALF, lung and blood samples were frozen and kept at minus 80°C until further processing.

Another series of animals were used for lung TBARS measurements. After anaesthesia and transection of the aorta and the inferior vena cava, the chest was opened and 10 mL of normal saline was injected through the right atrium with the heart still beating, the lungs becoming white as blood was flushed [25]. Samples from the left lower lobe were used for lung TBARS measurements.

Biochemical determinations

Haematocrit was measured in duplicate after centrifugation (Sigma 201M; Bioblock Scientific, Illkirch, France). Lipid peroxidation was measured as the level of TBARS expressed in nanomoles of malondialdehyde (MDA) in plasma (nmol MDA·mL⁻¹) and lung tissues (nmol MDA·mg⁻¹ protein) according to MAUPOIL and ROCHETTE [27]. cGMP was measured in BALF using a radioimmunoassay procedure (Amersham France SA, Les Ulis, France). The nitrate content of BALF was measured by ionic chromatography on a DX100 ion chromatograph (Dionex SA, Jouy en Josas, France). Known concentrations of nitrates were used to build dose-response curves in order to calibrate the equipment before each set of measures. Concentration of protein, potassium and LDH in BALF were measured on an automated analyser (Dax 48; Bayer Diagnostic, France).

Statistical analysis

Results are expressed as mean±SEM. The mortality analysis was performed using the Log Rank Test. The effects of oxygen exposure and of pretreatment with L-NAME were assessed using two-way analysis of variance (ANOVA), the two factors being oxygen and L-NAME, and the Student Newman-Keuls test was used as a *post hoc* test. A p-value of less than 0.05 was considered significant.

Results

Among the animals breathing high partial pressure of oxygen, L-NAME resulted in earlier death. As shown in figure 1, 57% of the O-LN rats died before 60 h *versus* 22% of O-S animals (p<0.01). At 60 h, the probability of death was 2.6 (relative risk (RR) 1.34–5.16) times higher in the L-NAME group. However, all the oxygen-exposed animals died within 4 days. On the other hand, no death occurred within 4 days in the two groups of air-breathing animals.

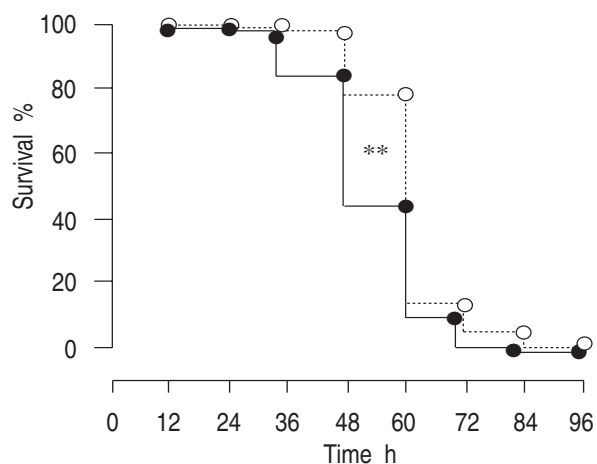
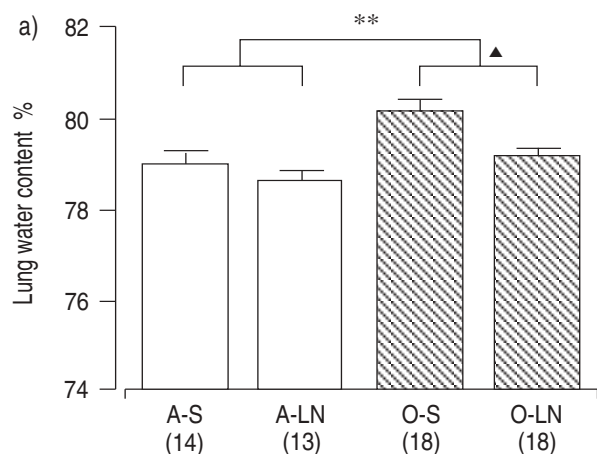


Fig. 1 - Occurrence of death in rats receiving intraperitoneal normal saline or N^G -nitro-L-arginine methyl ester (L-NAME) and breathing oxygen. ...O...: saline-treated rats (O-S); —●—: L-NAME treated rats (O-LN). Thirty seven animals were studied in both groups. Mortality was higher at 60 h in the O-LN than in the O-S (**: $p < 0.01$).



Lung water content (wet weight - dry weight/wet weight, expressed as percentage) was higher in the oxygen-breathing groups ($p < 0.01$), and was slightly decreased by administration of L-NAME ($p < 0.05$; O-S $80.1 \pm 0.29\%$; O-LN $79 \pm 0.29\%$; A-S $79 \pm 0.33\%$; and A-LN $78.5 \pm 0.34\%$) (fig. 2a). However, multiple comparison procedures showed that the L-NAME treatment induced a significant decrease only in the oxygen-exposed animals ($p < 0.05$). Oxygen exposure decreased lung TBARS ($p < 0.001$) (fig. 2b). In animals treated with L-NAME, there was a trend for lower lung TBARS than in normal saline rats, but this difference did not reach statistical levels ($p = 0.06$).

In contrast, blood TBARS were significantly elevated ($p < 0.01$) in animals which had inhaled oxygen, but L-NAME had no significant effect (fig. 3a). Haematocrit was increased by oxygen exposure ($p < 0.001$) and by L-NAME ($p < 0.001$) (fig. 3b).

Proteins in the BALF were increased by oxygen exposure ($p < 0.01$) and L-NAME treatment ($p < 0.01$) (fig. 4)

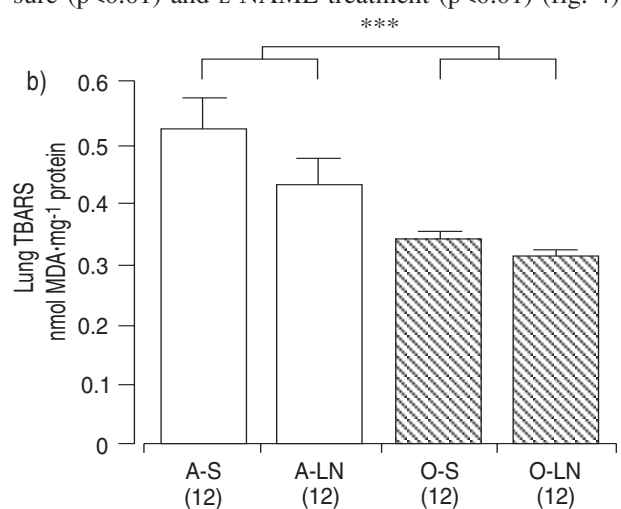


Fig. 2 - Effects of 36 h oxygen-breathing and L-NAME administration on the lungs of rats. Values are presented as mean \pm SEM. a) Lung water content was higher in the animals breathing oxygen and receiving normal saline (**: $p < 0.01$). L-NAME significantly reduced the lung water content only in the oxygen-exposed animals (Δ : $p < 0.05$ *post-hoc* analysis). b) Lung TBARS were significantly lower in the two groups which inhaled oxygen ($p < 0.001$). L-NAME decreased lung TBARS, but without reaching the significance level (2-way ANOVA, $p = 0.06$). O: oxygen; A: air; S: normal saline; LN: L-NAME. The number of animals in each series is indicated at the bottom of the columns. L-NAME: N^G -nitro-L-arginine methyl ester; TBARS: thiobarbituric acid reactive species; MDA: malondialdehyde.

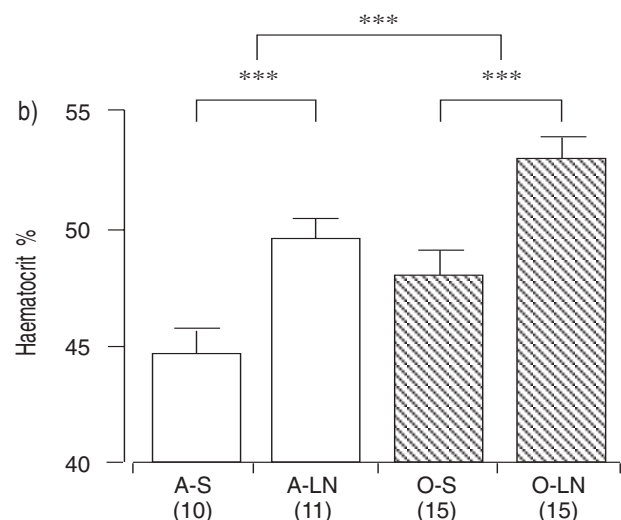
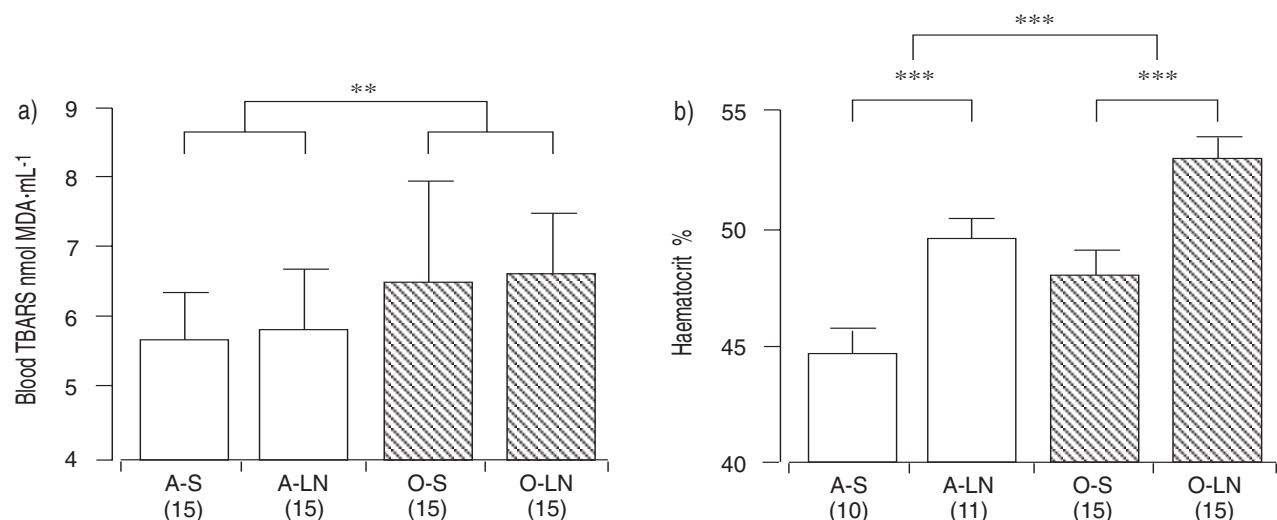


Fig. 3 - Blood effects of oxygen-breathing and L-NAME administration in rats. a) There was a significant increase in blood TBARS in rats of the two oxygen-exposed groups ($p < 0.01$). TBARS concentration is expressed as nmol MDA·mL⁻¹. b) Haematocrit level was greater both in oxygen-breathing rats ($p < 0.001$) and in animals which had received L-NAME ($p < 0.001$). O: oxygen; A: air; S: normal saline; LN: L-NAME. The number of animals in each series is indicated at the bottom of the columns. For definitions see legend to figure 2.

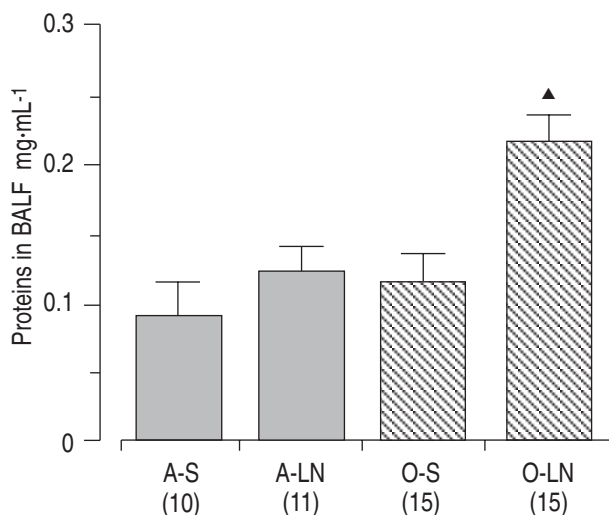


Fig. 4 – Plasma protein content of bronchoalveolar lavage fluid (BALF). The plasma protein content was higher in the rats breathing oxygen and injected with L-NAME than in the other three groups (\blacktriangle : $p < 0.05$ post-hoc analysis). O: oxygen; A: air; S: normal saline; LN: L-NAME. The number of animals in each series is indicated at the bottom of the columns. L-NAME: N^G-nitro-L-arginine methyl ester.

(A-S 0.093 ± 0.024 ; A-LN 0.12 ± 0.023 ; O-S 0.118 ± 0.02 ; and O-LN 0.215 ± 0.02). However, oxygen had no effect on the saline-treated rats, and L-NAME had no effect on the air-exposed animals. A significant increase was observed only between the O-LN group *versus* the O-S group and the A-LN group ($p < 0.05$). Concentrations of nitrates, cGMP, LDH and potassium in BALF were similar in the four groups (data not shown).

Discussion

The main results of this study were that, in rats continuously breathing high concentrations of oxygen, the inhibition of nitric oxide synthases with L-NAME treatment had a deleterious effect, with a decreased length of survival and increased vascular pulmonary permeability. Indeed, an unusually high early mortality rate was found in O-LN rats, whereas survival in the O-S group matched the usual observations. Recently, a decreased survival rate and a decreased tolerance to hyperoxia were also reported in newborn rats after administration of L-NAME to the dams during the final 7 days of gestation and during lactation [28]. L-NAME was used because it is a well-recognized inhibitor of constitutive and inducible NO synthases, which are both stimulated by hyperoxic exposure [8, 9]. However, the respective roles of constitutive and inducible NO synthases remain to be evaluated through the use of a specific inducible NOS inhibitor, such as aminoguanidine.

The observation of a higher protein content in the BALF of the O-LN rats after 36 h was contrasted with similar lower protein contents in the O-S and air-breathing animals, suggesting a toxic effect of the NO synthase inhibition by L-NAME. It is, in fact, unusual to find pathological features of pulmonary injuries after 36–48 h oxygen exposure [1]. Furthermore, our results are in accordance with a recent study demonstrating that inhalation of 40 parts per million (ppm) NO for less than 1 hour, decreases pulmonary transvascular albumin flux in patients

with acute lung injury [29]. In the O-LN group, the combination of a higher protein content in BALF and a larger haematocrit is strong circumstantial evidence of large plasma extravasation into the airway lumen, and it is considered to be a good appraisal of lesions of the alveolar barrier. Indeed, haematocrit has been directly correlated with the severity of interstitial oedema [1]. At the time the rats were sacrificed, oxygen toxicity had produced a significant interstitial oedema in the O-S group, as evidenced by the higher lung water content. Since the increase in lung water content precedes the increase of protein concentration in BALF [30], the decrease in the oxygen-induced elevation of the lung water content following L-NAME treatment could be consistent with a faster sequence of pulmonary injuries in O-LN rats. This decrease of the severity of pulmonary oedema following L-NAME treatment could also be related to a higher dehydration status.

Surprisingly, TBARS, considered as an index of lipid peroxidation, decreased in the lungs of the oxygen-breathing animals. This could be related to an increase of free radical defence mechanisms in response to oxygen aggression. In fact, it has been demonstrated that hyperoxia induced both an early increase of messenger ribonucleic acid (mRNA) antioxidant enzyme expression, and a later increase in superoxide dismutase, catalase and glutathione peroxidase activities [1, 11, 28]. On the other hand, the increase of plasma TBARS concentration in the oxygen-breathing groups could suggest that TBARS were released from the lungs to the blood after 36 h of oxygen exposure. We did not report a specific L-NAME effect on TBARS levels either in the plasma or in the lung. In another experimental model (smoke inhalation with concurrent inflammation in the rat), injection of L-NAME prevented the increase of plasma TBARS [26]. However, a recent study conducted with liposomes exposed to xanthine oxidase-derived reactive species demonstrated that the relative rates of production and steady-state concentrations of superoxide and nitric oxide profoundly influence expression of the oxidant-protective effect of NO [31]. Hence, for reactions mediated by NO and O₂⁻, it seems necessary to study the time course and the quantity of NO and O₂⁻ production as well as the antioxidant status of the tissue [32].

The higher oxygen toxicity in animals treated with L-NAME could be related to several mechanisms. Indeed, free radical toxicity, recruitment and activation of leucocytes, and vascular reactivity might be significantly changed by inhibition of NO synthesis. As previously determined, inhaled NO or NO donors decreased the deleterious effect of oxygen free radical [14, 16]. It could then be suggested that the decrease of NO production following L-NAME treatment may increase the oxygen-derived free radical toxicity. NO may also inhibit the formation of free radicals *via* the arachidonic acid pathway. Cyclo-oxygenase and lipoxygenase activities are stimulated by exposure to high oxygen concentrations and, in turn, their products increase lung accumulation of plasma albumin [9]. NO has an inhibitory effect on lipoxygenase and cyclo-oxygenase through its ability to reduce the active ferric enzymes to inactive ferrous forms [33].

During oxygen exposure, polymorphonuclear cells are recruited and activated early, and these cells contribute

substantially to tissue damage through the release of proteolytic enzymes, inflammatory mediators and free radicals. It has been recognized that NO inhibits neutrophil adherence and activation in different organs [17] and also prevents the production of oxygen free radicals by N-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol myristate acetate (PMA)-stimulated neutrophils [34]. Thus, NO, released in response to cytokines produced by endothelial cells or macrophages at sites of inflammation, may protect against neutrophil-dependent tissue injury [35]. In a model of lung ischaemia-reperfusion, a beneficial effect of inhaled NO has been reported and may result from the prevention of the inflammatory response associated with oxidant stress. Inhaled NO may positively affect the balance of oxidative reactions in the lung by decreasing the production of neutrophil-derived superoxide radicals [4].

L-NAME could also cause an alteration of pulmonary vascular tone and structure [28], increasing the pulmonary hypertension under oxygen exposure leading to death [2, 3, 28]. In fact, inhaled NO may protect against the development of pulmonary vascular resistance and prevent the decrease in endogenous NO release in ischaemia-reperfusion acute lung injury [4]. However, it has also been reported that the protective effect of inhaled NO against pulmonary oedema in isolated rat lung perfused with activated neutrophils is independent of vascular action [34].

To conclude, intraperitoneal administration of NG-nitro-L-arginine methyl ester hastened pulmonary lesions, as demonstrated by a larger haematocrit, an increased protein content in bronchoalveolar lavage fluid and a decreased survival time of rats exposed continuously to high oxygen concentrations. Therefore, this deleterious effect might suggest that endogenous nitric oxide has a protective role against hyperoxia-induced pulmonary lesions. In addition, our results provide some evidence supporting the putative protective effect of inhaled nitric oxide in the treatment of severe acute respiratory distress syndrome.

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