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Nitric Oxide Mediates Lung Injury Induced by Ischemia-Reperfusion in Rats

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Key Words
Filtration coefficient · Ischemia · Lung injury · Nitric oxide · Reperfusion

Abstract
Nitric oxide (NO) has been reported to play a role in lung injury (LI) induced by ischemia-reperfusion (I/R). However, controversy exists as to the potential beneficial or detrimental effect of NO. In the present study, an in situ, perfused rat lung model was used to study the possible role of NO in the LI induced by I/R. The filtration coefficient (K_{fc}), lung weight gain (LWG), protein concentration in the bronchoalveolar lavage (PCBAL), and pulmonary arterial pressure (PAP) were measured to evaluate the degree of pulmonary hypertension and LI. I/R resulted in increased K_{fc}, LWG, and PCBAL. These changes were exacerbated by inhalation of NO (20–30 ppm) or 4 m*M* *L*-arginine, an NO precursor. The permeability increase and LI caused by I/R could be blocked by exposure to 5 m*M* N^{ω}-nitro-*L*-arginine methyl ester (*L*-NAME; a non-specific NO synthase inhibitor), and this protective effect of L-NAME was reversed with NO inhalation. Inhaled NO prevented the increase in PAP caused by I/R, while *L*-arginine had no such effect. *L*-NAME tended to diminish the I/R-induced elevation in PAP, but the suppression was not statistically significant when compared to the values in the I/R group. These results indicate that I/R increases K_{fc} and promotes alveolar edema by stimulating endogenous NO synthesis. Exogenous NO, either generated from *L*-arginine or delivered into the airway, is apparently also injurious to the lung following I/R.

Introduction

Ischemia-reperfusion (I/R) lung injury (LI) is an important phenomenon that arises in many clinical situations. It has been observed in lung transplantation [20] and thrombolysis after pulmonary embolism [23], and in extreme cases, it may lead to acute respiratory distress syndrome [12]. I/R injury results from a temporary interruption of blood flow to an organ, followed by reperfusion of the previously ischemic area [12].

Despite intense research, the mechanism of I/R injury has not yet been fully elucidated. Many reports have indicated that the compound nitric oxide (NO) may play a role in the development of I/R injury. However, agreement has not been reached concerning whether NO is

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beneficial or injurious to lung tissue after I/R injury. Some evidence has indicated that NO inhalation or excessive endogenous NO formation can be detrimental to lung tissue [10, 24]. For example, inhaled NO has been shown to prime alveolar macrophages to release reactive oxidants in Balb/c mice [24]. Inhaled NO also causes neutrophil activation and increases the level of 3-nitrotyrosine, a marker for peroxynitrite formation, in bronchoalveolar lavage (BAL) fluid in patients with the acute respiratory distress syndrome [10]. Inhibition of NO synthase (NOS) has also been shown to attenuate the acute LI produced by lipopolysaccharides [11, 15], as well as I/R-related injuries in the hindlimb, myocardium, and lung [8, 13, 17]. On the other hand, some reports have indicated that the administration of exogenous NO or NO donors in I/R injury may exert a therapeutic effect [16]. NO has been postulated to attenuate I/R injury by inhibiting neutrophil adhesion to the endothelium and by scavenging reactive oxygen species [9, 25].

In the present study, we have further explored the role of NO in I/R LI by evaluating the effects of inhaled NO, an NO precursor (*L*-arginine) and an NOS inhibitor (N^{ω}-nitro-*L*-arginine methyl ester, *L*-NAME) on changes in the pulmonary filtration coefficient (K_{fc}) and pulmonary arterial pressure in an in situ, perfused rat lung model.

Methods

Isolation and Perfusion of Rat Lungs

Male Sprague-Dawley rats (300–350 g, specific pathogen free) were purchased from the National Animal Center and housed in a controlled environment at 22 ± 1 °C under a 12:12-hour light/dark cycle. Food and water were available ad libitum. The care and use of these animals were in accordance with the principles of the National and University Animal Centers.

The procedure used to prepare isolated, perfused lungs was similar to that described previously [6, 18, 22]. Rats were tracheotomized under pentobarbital anesthesia (30 mg/kg i.p.), and the lungs were artificially ventilated with room air supplemented with 5% CO_2. A midsternal thoracotomy was performed. Heparin (1 U/g) was administered intravenously, and 10 ml of blood was collected from the right ventricle and mixed with 10 ml of Hank's balanced salt solution (in m*M*: NaCl, 136.9; KCl, 5.4; glucose, 5.6; KH_2PO_4, 0.4; Na_2HPO_4, 0.3 + albumin 6%). The mixture was used for perfusing isolated lungs. A cannula was placed into the pulmonary artery through a puncture in the right ventricle, and a tight ligature was tied around the main trunk of the pulmonary artery and aorta. A large catheter was then inserted into the left atrium via the left ventricle and mitral valve and fixed by ligature to the apex of the heart, diverting the pulmonary venous outflow into a reservoir. A third ligature was placed above the ventricular junction to prevent flow of perfusate into the ventricles. Warmed (37 ± 0.5 °C) perfusion fluid was circulated using a roller pump at a flow rate of 8 ml/min. Pulmonary arterial pressure (PAP) and pulmonary venous pressure (PVP) were measured with pressure transducers (Gould Instruments, Cleveland, Ohio, USA) from a side arm of the inflow and outflow cannulae, respectively. The PVP was set at 3 cm H_2O by adjusting the height of the venous reservoir.

Lungs were ventilated at 70 breaths/min after initial hyperventilation. The tidal volume was 2 ml, and the end-expiratory pressure was set at 2 cm H_2O. Preparations that remained in situ exhibited no leakage at the cannula insertion sites or evidence of edema, and were in an isogravimetric state. The weight of the whole rat was monitored on an electronic balance and was recorded on an oscillograph. Changes in body weight (BW) were considered to be the result of changes in lung weight (LW). Our earlier examinations of isolated lung preparations in which Evans blue dye was added to the perfusate revealed that the dye was confined to the lungs after exposure to either low (2.5 mm Hg) or high (10 mm Hg) PVP for 30 min [18, 22]. Although the hematocrit was reduced in the perfusate, the perfusate osmolarity (285 mosm) was not significantly altered compared to that of whole blood. Moreover, the stability of the perfusion system was tested in many preparations in which PAP and LW remained essentially constant for 60 min after an initial adjustment of the flow rate to set the PAP at 15–20 cm H_2O [6, 22].

Induction of Lung I/R

I/R was induced in lungs essentially as previously described [6]. Lungs were initially ventilated with 5% CO_2-95% N_2 for 10 min to decrease the O_2 content in the perfusate. Thereafter, ventilation and perfusion were stopped for 95 min. To facilitate subsequent reperfusion, the lungs were kept inflated during this 95 min of ischemia by holding the ventilation in an inspiration state. After the ischemia, the lungs were reperfused and ventilated with 5% CO_2-95% air for 50 min.

Administration of NO Gas

NO was delivered to the lungs via a ventilator. The levels of NO in the inhaled gas (Air Product, Allen, Calif., USA) were monitored using a Micro Medical Gas System (Micro Medical Limit, Bethany, Okla., USA) and maintained at approximately 20 ppm.

Measurement of LW and K_{fc}

All experiments were terminated after 155 min of closed extracorporeal perfusion, and the lungs were then removed and weighed. Because the initial LW could not be obtained in animals subjected to the experimental protocols, the relation between LW and BW was determined in preliminary experiments [6, 22]. Both BW and LW were measured in 30 control animals sacrificed by decapitation. LW was plotted as a function of the corresponding BW to obtain the following equation: LW (g) = 0.005 BW (g) + 0.0015. This equation was used to estimate the initial LW in all experimental animals.

K_{fc} as an index of microvascular permeability was calculated from the increase in LW produced by an elevation in PVP. The K_{fc} was defined as the initial weight gain rate (g/min) divided by PVP (10 cm H_2O) and LW, and expressed as g/min/cm H_2O/100 g [18]. During the experiment, PVP was rapidly elevated by 10 cm H_2O for 7 min to measure K_{fc}. This hydrostatic challenge elicited a biphasic increase in LW: an initial rapid rise, followed by a slow and steady increase that began about 2 min after the onset of PVP elevation. By plotting the log of the weight gain as a function of time, the initial transcapillary filtration rate was obtained by extrapolating the slow component of the weight gain back to time 0 [18, 22].

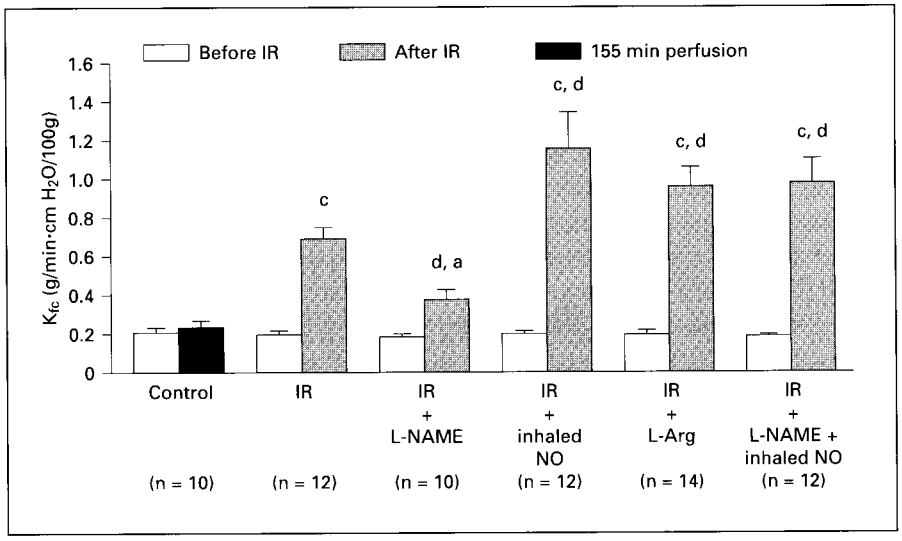

Fig. 1. Effect of I/R on K_{fc}. Isolated, perfused lungs were challenged by I/R in the presence or absence of *L*-NAME, *L*-arginine (L-Arg), or inhaled NO. Data are presented as means ± SEM. [a] $p < 0.05$, [c] $p < 0.001$ vs. the control group; [d] $p < 0.05$ vs. the I/R group.

Measurement of Protein Concentration in the BAL

After the experiment, lungs were lavaged twice with saline (2.5 ml/lavage). Lavage samples were centrifuged at 1,500 *g* at room temperature for 10 min. The protein concentration in BAL (PCBAL) in the supernatant was determined with a spectrophotometer by measuring the change in absorbance at 630 nm after the addition of bromocresol green [18, 22].

Drugs

L-NAME and *L*-arginine were purchased from Sigma (St. Louis, Mo., USA). These agents were dissolved in saline solution immediately before use.

Experimental Protocol

In all preparations, PAP and LW remained essentially constant for at least 10 min after the perfusion flow was adjusted to set the PAP at 15–20 cm H_2O. The baseline K_{fc} was determined during this steady-state period. After K_{fc} measurement, LW gain (LWG) gradually returned to baseline in all groups. Then, in the control group (n = 10) which received no I/R challenge, only continuous perfusion and ventilation were provided for 155 min. Lungs in the I/R group (n = 12) were subjected to 10 min of ventilation with 5% CO_2-95% N_2, then 95 min of ischemia, followed by 50 min of reperfusion. In the other four groups, NO (20–30 ppm; n = 12), *L*-arginine (4 m*M*, n = 14), *L*-NAME (5 m*M*, n = 10), or *L*-NAME with inhaled NO (n = 12) was given during the reperfusion period. Since the drug treatments by themselves did not change the K_{fc} or LWG, we did not include these data in the Results section.

Data Analysis

Values are expressed as means ± SEM. Comparisons of K_{fc} values among groups were made using a paired t test. Comparisons of PCBAL values among groups were made using one-way analysis of variance and Scheffé's comparison a posteriori. Comparisons of LWG and PAP changes among groups were made using multiple factors with repeated measures (a generalized estimation equation) model. Values of $p < 0.05$ were considered statistically significant.

Results

Pulmonary Filtration Coefficient

The K_{fc} was essentially unaffected during 155 min of perfusion in the control group (fig. 1). I/R produced a 3- to 4-fold increase in K_{fc}. Inhalation of NO during the 50-min reperfusion period in the I/R group significantly augmented the increase in K_{fc} ($p < 0.001$). Increasing the availability of NOS substrate by administration of *L*-arginine augmented the I/R-induced increase in K_{fc} ($p < 0.001$). Inhibition of NOS by *L*-NAME greatly blocked the increase in K_{fc} elicited by I/R ($p < 0.05$). However, cotreatment of *L*-NAME with inhaled NO reversed the attenuating effect of *L*-NAME ($p < 0.001$).

LWG during Reperfusion

The effects of I/R on LWG paralleled those on K_{fc}, indicating that the observed increase in K_{fc} caused excessive edema. Under control conditions, LW remained unchanged during the entire perfusion period (fig. 2, ●). However, during reperfusion after ischemia, a biphasic increase in LW occurred (fig. 2, ○). First, the lung rapidly gained in weight during the first 5 min of reperfusion due to an influx of perfusate. Thereafter, the LW increased slowly over the next 45 min, reflecting edema development. After 50 min of reperfusion, LW increased from a baseline value of 0.14 ± 0.10 to 2.31 ± 0.27 g ($p < 0.001$). Consistent with its effects on K_{fc}, *L*-NAME also significantly attenuated the I/R-induced LWG ($p < 0.001$) during the reperfusion period (fig. 2, □). Neither inhaled NO (fig. 2, ■) nor *L*-arginine (fig. 2, △) significantly affected

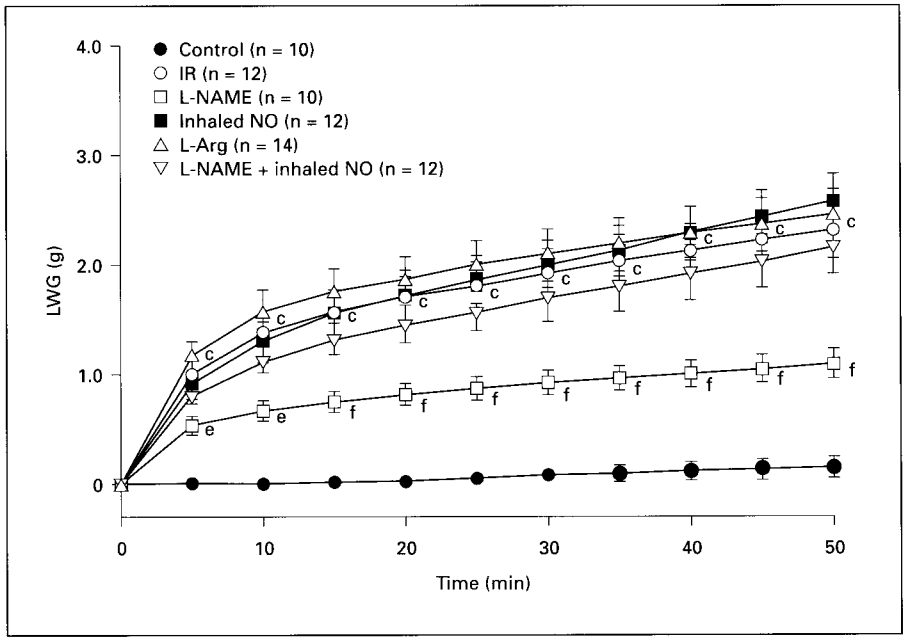

Fig. 2. Effect of I/R on LWG. The graphs plot the time-dependent increase in LWG induced by I/R in the presence or absence of the indicated agents. Note that LW remained unchanged in the control group and was attenuated in I/R when *L*-NAME was added. Data are presented as means ± SEM. [c] $p < 0.001$ vs. control group; [e] $p < 0.01$, [f] $p < 0.001$ vs. I/R group.

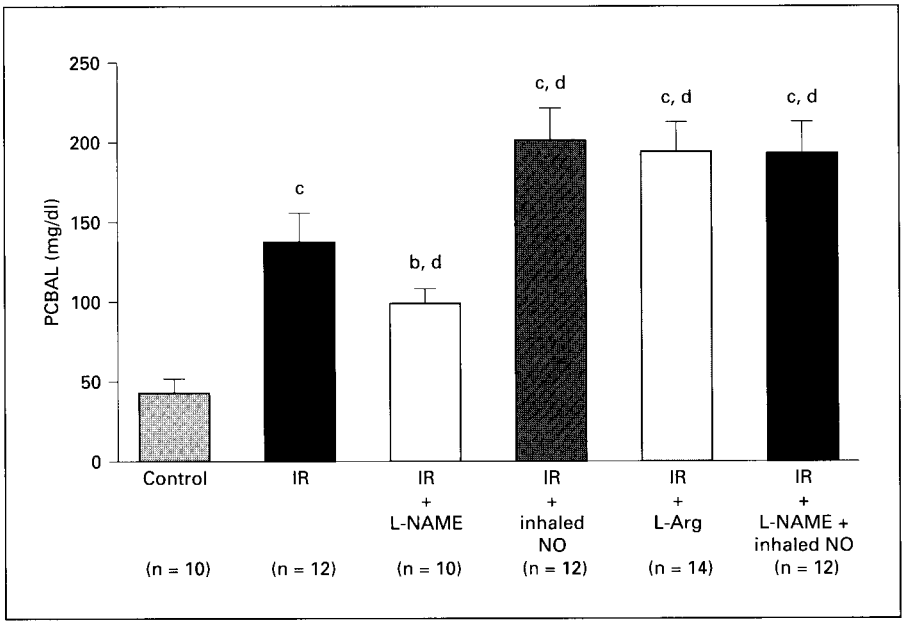

Fig. 3. Effect of I/R on PCBAL in the presence or absence of the indicated agents. [b] $p < 0.01$, [c] $p < 0.001$ vs. the control group; [d] $p < 0.05$ vs. the I/R group.

the LWG during the reperfusion period. Interestingly, inhaled NO reversed the inhibitory effect of *L*-NAME (fig. 2).

Since the accumulation of fluid in the lung was associated with an increase in permeability to protein, I/R significantly ($p < 0.001$) increased PCBAL (fig. 3). The increase in PCBAL following I/R was augmented by NO inhalation or *L*-arginine administration ($p < 0.05$) but was attenuated by *L*-NAME ($p < 0.05$). Coadministration of NO with *L*-NAME increased the PCBAL ($p < 0.01$). In addition, *L*-NAME with inhaled NO enhanced the effect of I/R ($p < 0.05$).

PAP Changes following I/R

After ischemia and at the onset of reperfusion, the PAP increased rapidly from a baseline of 0.25 ± 0.31 to 9.75 ± 0.69 cm H_2O ($p < 0.001$; fig. 4, ○, time 0). After ischemia, reperfusion of the lungs caused the PAP to decline

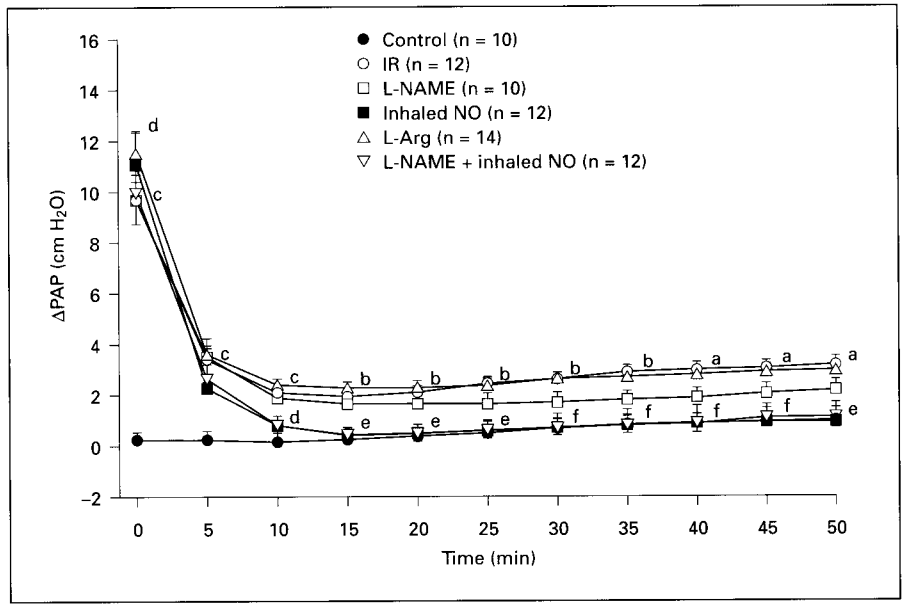

Fig. 4. Effect of I/R on changes in PAP. The graphs plot the time-dependent changes in PAP evoked during reperfusion for 50 min in the presence or absence of the indicated agents. Note that the PAP was unchanged in the control group. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ vs. the control group; [e] $p < 0.05$, [f] $p < 0.01$, [g] $p < 0.001$ vs. the I/R group.

dramatically; however, it remained significantly higher than control values (fig. 4, ●). During the late phase of reperfusion (20–50 min), there was a slight tendency for the PAP to increase again, and ultimately it rose to 3.20 ± 0.36 cm H_2O after 50 min of reperfusion. The increase in PAP was significantly higher than that for the control group (1.07 ± 0.71 cm H_2O, $p < 0.05$).

When inhaled NO was given at the onset of reperfusion, the PAP declined to baseline levels, and the late increase in PAP was also blocked (fig. 4, ■). *L*-arginine had no attenuating effect on the PAP increase during reperfusion (fig. 4, △). *L*-NAME tended to decrease the I/R-induced increase in PAP (fig. 4, □), but the suppression was not statistically significant when compared to values in the I/R group. When exogenous NO was given simultaneously with *L*-NAME (▽), the effect was virtually identical to that seen with inhaled NO alone.

Discussion

In the present study, we evaluated the extent of I/R-induced LI in an in situ rat lung model by measuring K_{fc}, LWG, and alveolar protein leakage. The results indicated that in this model, NO inhalation aggravated I/R-induced LI, as did *L*-arginine. The NO synthase inhibitor *L*-NAME attenuated LI, and its attenuation effect was reversed by the addition of inhaled NO. Thus, our findings indicate that endogenous and exogenous NO are deleterious to I/R-induced tissue injury. The attenuating effect of *L*-NAME may be related to its inhibition of endogenous NO production, which would decrease hydroxyl radical and peroxynitrite production.

Administration of exogenous NO by inhalation reversed the effect of *L*-NAME and increased the extent of injury, possibly because inhaled NO increased the NO concentration to a much greater extent than did endogenous NO production, which thereby reversed the protective effect of *L*-NAME.

Our results are in agreement with several reports which found that NO causes damage to the myocardium in piglets subjected to hypoxia-reoxygenation [13], in rat brains after hypoxia-reoxygenation [2], and in rat kidney tubules after hypoxia-reoxygenation [26]. Chang et al. [3] demonstrated that the *L*-arginine-NO pathway contributes to LI induced by hyperoxia, and Tomita et al. [21] also showed that an NO donor has deleterious effects in a lung carcinoma cell line. In contrast, the production of NO has been shown to be protective in tissue injury [1, 5, 19, 27]. NO donors and *L*-arginine reduced myocardial necrosis and endothelial dysfunction associated with cardiac I/R in cats [19]. Furthermore, in a rat model of global brain I/R, NO synthase inhibition raised the extracellular glutamate concentration and increased the extent of cytotoxicity [27]. Inhaled NO was also able to maintain gas exchange in a setting of I/R-induced ventilation-perfusion mismatch [5], and low-dose NO inhalation was found to enhance rat lung graft function [1].

In the lungs, ischemia-reperfusion causes microvascular damage, an increase in capillary permeability, and acute pulmonary edema [6, 14]. Previous efforts to elucidate the role of NO in I/R-induced LI have produced variable results, depending on the experimental conditions and timing of the I/R. Eppinger et al. [4] reported disparate effects of NO on I/R-induced LI. They found that inhaled NO (80 ppm) was toxic in the early stages of perfusion, presumably because of the release of peroxynitrite from NO and superoxide, but it became beneficial after longer reperfusion because of NO-mediated vasodilation. In isolated, blood-perfused rat lungs, Lu et al. [12] demonstrated that endogenous NO was protective against I/R-induced LI in conditions of ischemia for 30 min and reperfusion for 180 min in normal rats, and under ischemia for 30 min and reperfusion for 30 min in endotoxin-treated rats.

The present study reveals that inhalation of even a low dose of NO (20–30 ppm) can exacerbate I/R-induced LI in lungs subjected to hypoxia (95% N_2 and 5% CO_2 ventilation) before ischemia. Inhalation of NO also reversed the protective effect of *L*-NAME on I/R-induced LI. In this connection, Huang et al. [7] have suggested that O_2 tension in the lungs might alter NO biosynthesis and metabolism. They found that *L*-NAME exacerbated the I/R-induced LI in isolated rabbit lungs ventilated with air (21% O_2) during 90 min of ischemia and with 21% O_2 during 40 min of reperfusion. In contrast, when lungs were ventilated with 95% N_2 during a 90-min ischemia and with 21% O_2 during a 40-min reperfusion, *L*-NAME was able to attenuate I/R-induced LI. Their interpretation was that hypoxia prevented the protective effects of NO on I/R-LI, which may be related to lower NO production as a result of oxidant stress during IR. Hypoxia may also alter the metabolic rate of NO-mediated production of peroxynitrite, which is thought to be toxic to I/R lungs [8]. It is known that xanthine oxidase and oxygen radicals are involved in tissue injury following I/R. When the alveolar O_2 tension is low, I/R tends to produce more free radicals, including superoxide, hydrogen peroxide, and peroxynitrite. As a result, exogenous NO may become toxic to the lungs following I/R. It should be noted that the lungs were only preconditioned with a 10-min hypoxic exposure in the present study. This short period of hypoxic challenge before I/R produced effects similar to those obtained by Huang et al. [7].

After I/R in the lungs, the late-phase increase in PAP that occurs during reperfusion indicates that vasoconstrictors may be involved. A previous study [14] suggested that thromboxane A_2 is one of the mediators contributing to vasoconstriction. In the present experiment, we observed that the late-phase PAP increase was not affected by either *L*-arginine or *L*-NAME. In contrast, the late vasoconstrictory response was abolished by inhalation of NO, with or without *L*-NAME (fig. 4). Possible explanations for these findings are that inhaled NO itself caused vasodilation or that exogenous NO mediated the release of more vasodilators than vasoconstrictors. The results also suggest that a late vasoconstrictory response to I/R is not a major factor in I/R-induced LI.

In summary, we demonstrate in this study that NO inhalation is damaging to the lungs following I/R. I/R increases K_{fc} and promotes alveolar edema by stimulating endogenous NO synthesis. Exogenous NO, either generated from *L*-arginine or delivered into the airway, is injurious to the lungs following I/R.

Acknowledgments

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