The PDE inhibitor zaprinast enhances NO-mediated protection against vascular leakage in reperfused lungs

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The PDE inhibitor zaprinast enhances NO-mediated protection against vascular leakage in reperfused lungs. Am J Physiol Lung Cell Mol Physiol 279: L496–L502, 2000.—Disruption of endothelial barrier properties with development of noncardiogenic pulmonary edema is a major threat in lung ischemia-reperfusion (I/R) injury that occurs under conditions of lung transplantation. Inhaled nitric oxide (NO) reduced vascular leakage in lung I/R models, but the efficacy of this agent may be limited. We coadministered NO and zaprinast, a cGMP-specific phosphodiesterase inhibitor, to further augment the NO-cGMP axis. Isolated, buffer-perfused rabbit lungs were exposed to 4.5 h of warm ischemia. Reperfusion provoked a transient elevation in pulmonary arterial pressure and a negligible rise in microvascular pressure followed by a massive increase in the capillary filtration coefficient and severe lung edema formation. Inhalation of 10 parts/million of NO or intravascular application of 100 μM zaprinast on reperfusion both reduced pressor response and moderately attenuated vascular leakage. Combined administration of both agents induced no additional vasodilation at constant microvascular pressures, but additively protected against capillary leakage paralleled by a severalfold increase in perfusate cGMP levels. In conclusion, combining low-dose NO inhalation and phosphodiesterase inhibition may be suitable for the maintenance of graft function in lung transplantation by amplifying the beneficial effect of the NO-cGMP axis and avoiding toxic effects of high NO doses.

phosphodiesterase; nitric oxide; lung transplantation; cyclic nucleotides; microvascular permeability

LUNG ISCHEMIA-REPERFUSION (I/R) injury can adversely affect graft function in the early period after lung transplantation, provoking high-permeability edema, increased pulmonary vascular resistance, and impairment of gas exchange (7, 38), and may lower survival (1). Inhalation of nitric oxide (NO) has been proposed as a therapeutic approach in lung allograft recipients suffering from respiratory failure (25). NO is known to improve ventilation-perfusion matching and oxygenation in models of acute lung injury and in clinical acute respiratory distress syndrome by acting as a selective pulmonary vasodilator (44). Beyond this, NO was suggested to be protective against pulmonary edema formation by preserving microvascular barrier function via nonvasodilatory anti-inflammatory mechanisms (13, 32). In experimental settings of I/R-induced lung leakage, several studies (4, 6, 16, 27) have shown beneficial effects of exogenous NO, but lack of protection (30) or worsening of edema (11) has also been reported. NO efficacy in lung I/R may thus critically depend on the dose and timing of inhalation (6, 28). Moreover, even an optimum regimen of inhalational NO administration may possess limited efficacy under conditions of most severe injury due to prolonged ischemia as recently reported (16).

Several biological actions of NO are mediated via the activation of soluble guanylate cyclase, resulting in an enhanced appearance of cGMP. cGMP is known to induce smooth muscle relaxation (44) and may reduce microvascular permeability by some direct action on endothelial cells (10, 43). Moreover, inhibition of injurious leukocyte-endothelial interactions may also be involved in endothelial protection mediated by cGMP (19, 22). Administration of the cGMP analog 8-bromo-cGMP (8-BrcGMP) in a rat lung transplantation model was demonstrated to attenuate reperfusion injury (31). In line with these notions, zaprinast, an inhibitor of cGMP-specific phosphodiesterase (PDE) type V, has been demonstrated to decrease pulmonary arterial pressure (Ppa) (5) and to reduce microvascular leak (34). Moreover, zaprinast and NO may have additive effects because zaprinast enhanced and prolonged the hemodynamic impact of inhaled NO in the pulmonary circulation in a model of pulmonary hypertension (18, 40).

In the present study, I/R injury was elicited in perfused rabbit lungs previously characterized in detail for this type of injury (16, 35). The duration of the warm ischemic period was prolonged to 4.5 h to provoke a massive microvascular permeability increase and edema formation. Under these conditions, even an optimum regimen of NO inhalation, with the gaseous...
agent being administered throughout the reperfusion period, exerted only moderate protection against the leakage response, and this was similarly true for the PDE inhibitor zaprinast applied in a sufficiently high dose. However, coadministration of inhaled NO and the PDE inhibitor resulted in impressive protection against the pulmonary leakage response in the reperfusion period. Further data analysis supports the view that this effect of NO-zaprinast coadministration is related to an enhanced appearance of cGMP and is independent of the pulmonary vasodilatory efficacy of this approach. The present findings thus further strengthen the concept of employing the NO-cGMP axis for lung protection in I/R.

MATERIALS AND METHODS

Reagents. Sterile Krebs-Henseleit-hydroxyethylamyllopectin buffer was obtained from Serag-Wiessner (Naila, Germany). The buffer contained 120 mM NaCl, 4.3 mM KCl, 1.1 mM KH₂PO₄, 24 mM NaHCO₃, 2.4 mM CaCl₂, 1.3 mM MgCl₂, and 2.4 g/l of glucose as well as 5% (wt/vol) hydroxyethylamyllopectin (mol wt 200,000) as an oncotic agent. NO (in pure nitrogen), the gas mixture for anoxic ventilation (95% N₂-5% CO₂), as well as all O₂, CO₂, and N₂ for the gas mixing chamber were obtained from Messer Griesheim (Herborn, Germany). Zaprinast (M & B 22948) was a generous gift from Rhone-Poulenc Rorer (Dagenham, UK).

Lung model. The technique of isolated rabbit lung perfusion has been previously reviewed (36). Briefly, rabbits of either sex weighing 2.5–3.1 kg were anticoagulated with 1,000 U/kg of heparin and deeply anesthetized with ketamine and xylazine. A tracheostomy was performed, and the animals were room air ventilated with a Harvard respirator (cat/rabbit ventilator; Hugo Sachs Elektronik, March-Hugstetten, Germany) with a tidal volume of 30 ml, a frequency of 30 breaths/min, and a positive end-expiratory pressure of 1 cmH₂O. After a midsternal thoracotomy, catheters were inserted into the pulmonary artery and left atrium, and perfusion with sterile Krebs-Henseleit-hydroxyethylamyllopectin buffer was started. Sterilized perfusion circuit tubing was used throughout. In parallel with the onset of artificial perfusion, the gas supply was changed to a mixture of 5% CO₂-21% O₂-74% N₂ provided by a gas mixing chamber (Witt, Witten, Germany). For the washout of blood, the perfusate was initially not recirculated. The lungs were removed from the thorax without interruption of ventilation and perfusion and were freely suspended from a force transducer for the monitoring of organ weight in a temperature-equilibrated, humidified chamber at 37.5°C. In a recirculating system, the flow was slowly increased to 100 ml/min (total volume 150 ml). Left atrial pressure was set at 2.5 mmHg (referred to the hilum), and the whole perfusion system was equilibrated at 37.5°C. Additionally, the inspiration loop of the ventilation system was connected to a humidifier and heated to 37.5°C.

Ppc and pulmonary venous pressure (Ppv) were monitored with pressure transducers and digitized with an analog-to-digital converter, thus allowing data sampling with a personal computer. The microvascular (pulmonary capillary) pressure (Ppc) was determined by the arterial and venous double-occlusion technique. Electromagnetic tube clamping devices were used for the simultaneous interruption of arterial and venous flows in end expiration, and the mean Ppc was calculated with a spreadsheet program (Microsoft Excel) from Ppc and Ppv values after double occlusion. The capillary filtration coefficient (Kfc) and total vascular compliance were determined gravimetrically from the slope of the lung weight gain curve induced by a 7.5-mmHg step elevation of the venous pressure for 8 min as previously described (36). Lung weight gain was calculated as the difference in organ weight measured directly before and 5 min after each of these pressure elevation maneuvers. Vascular compliance was calculated from the initial steep increase in lung weight on a step change in Ppc.

Inclusion criteria for the study were 1) a homogeneous white appearance of the lungs with no signs of edema, hemothrosis, or atelectasis; 2) initial Ppa and ventilation pressure values in the normal range; and 3) constancy of organ weight during an initial steady-state period of at least 20 min.

Inhalation of NO. NO was admixed to the inspiratory gas flow with the use of a gas mixing chamber while keeping the inspiratory O₂ and CO₂ concentrations constant. The concentration of NO in the inspired gas was controlled by a chemiluminescence detector (UPK 3100, UPK, Bad Nauheim, Germany).

Measurement of cGMP. cGMP was determined in samples of pulmonary venous effluent before ischemia as well as 15, 30, 60 and 90 min after the onset of reperfusion. Samples were analyzed with a commercially available RIA (Beckman-Coulter, Hamburg, Germany).

Experimental protocols. After termination of the initial steady-state period and performance of a control hydrostatic challenge, time was set at zero and the lungs were exposed to ischemia by stopping the perfusion. The arterial and venous catheters were both clamped for maintenance of a positive intravascular pressure, which was initially adjusted to 6 mmHg. During ischemia, the lungs were continuously ventilated with a warmed and humidified O₂-free gas mixture (95% N₂-5% CO₂). At the end of ischemia, ventilation was changed to normoxia, and perfusion was reestablished by increasing the flow stepwise over 3 min. Hydrostatic challenges were performed 30, 60, and 90 min after the onset of reperfusion. Double-occlusion maneuvers for the assessment of Ppc were performed before ischemia as well as 3, 30, 60, and 90 min after reperfusion. Lungs were treated according to one of the following protocols: 1) ischemia: the lungs were exposed to 270 min of ischemia, and on reperfusion, no interventions were performed; 2) ischemia plus NO: after 270 min of anoxic ischemia, 10 parts/million (ppm) of NO were admixed to the inspiration gas immediately before the onset of reperfusion and the NO supply was continued until the termination of experiments; 3) ischemia plus zaprinast: after 270 min of anoxic ischemia, zaprinast (100 μM) was admixed to the perfusion buffer; 4) ischemia plus NO plus zaprinast: 10 ppm of NO were inhaled as in the ischemia plus NO group along with the intravascular application of 100 μM zaprinast; and 5) control: control lungs were perfused and normoxia ventilated without interruption of flow, and Kfc as well as Ppc was measured at time points corresponding to the ischemia experiments.

Each group encompassed five to six independent experiments (Table 1). All experiments were terminated after 90 min of reperfusion or when lung weight gain exceeded 25 g during reperfusion.

Data analysis. Data are expressed as means ± SE. Differences were analyzed by one-way analysis of variance followed by a post hoc Student-Newman-Keuls test. If necessary, values were log transformed to achieve a normal distribution before statistical analysis. P values < 0.05 were considered to represent a significant difference.


**RESULTS**

$P_{pa}$. In control experiments, $P_{pa}$ values were virtually constant throughout the entire experimental period (Fig. 1). When lungs were exposed to 270 min of anoxic ischemia, a transient $P_{pa}$ rise was noted on reperfusion, with, at most, doubling of $P_{pa}$ values. In lungs treated with inhaled NO, the pressor response was attenuated. Zaprinast reduced the pressor response to a similar extent; however, the combined application of both agents did not further suppress the $P_{pa}$ rise compared with each single agent (Fig. 1).

$P_{pc}$ and vascular compliance. $P_{pc}$ values were only marginally increased 3 min after reperfusion in untreated ischemic lungs and rapidly returned to baseline values (Table 1). Overall, there were only very minor variations between the different experimental groups, indicating a negligible contribution of the capillary filtration pressure to the edema formation encountered on reperfusion. Values of vascular compliance did not differ between control lungs and the different experimental groups (data not given in detail). The intravascular pressure, which was initially adjusted to 6 mmHg, slowly declined during the ischemic period to ~2 mmHg in all groups when measured at the end of ischemia (Table 1).

$K_{fc}$ and weight gain. After 270 min of ischemia, untreated ischemic lungs displayed dramatically elevated $K_{fc}$ values on reperfusion compared with non-ischemic lungs (Fig. 2). In parallel, massive edema formation was noted (Table 1), and experiments had to be discontinued after the 30-min measurement of $K_{fc}$ due to dramatic fluid accumulation. In the presence of either inhaled NO or only zaprinast, the $K_{fc}$ was strongly elevated, with marked increases in organ weight. However, the values were reduced compared with those in untreated ischemic lungs. When both NO and zaprinast were administered, a highly significant, additive effect on the microvascular leakage response was observed (Fig. 2, Table 1). In contrast to all other ischemia experiments, a complete 90-min postischemic observation period could be performed in this group.

cGMP release. In untreated ischemic lungs, cGMP levels did not increase on reperfusion. In lungs exposed to inhaled NO or intravascular zaprinast alone, a significant increase in intravascular cGMP was measured during the reperfusion period (Fig. 3). When both NO and zaprinast were administered, a severalfold elevation in cGMP was observed, again indicating a strong additive effect of both agents (Fig. 3). In control lungs with ongoing perfusion, a continuous accumulation of cGMP during 270 plus 90 min of perfusion was observed (data not given in detail).

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**Table 1. Microvascular pressure and lung weight gain during ischemia-reperfusion**

<table>
<thead>
<tr>
<th>Conditions During Ischemia</th>
<th>Duration, min</th>
<th>$F_{O_2}$</th>
<th>End-ischemic intravascular pressure, mmHg</th>
<th>Weight Gain, g</th>
<th>Microvascular Pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>Ischemia</td>
<td>Before ischemia</td>
<td>30 min</td>
<td>60 min</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>270</td>
<td>0.21</td>
<td>1.3±0.3</td>
<td>3.4±0.8</td>
</tr>
<tr>
<td>Ischemia</td>
<td>6</td>
<td>270</td>
<td>0</td>
<td>1.9±0.4</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Ischemia + NO</td>
<td>5</td>
<td>270</td>
<td>0</td>
<td>2.9±0.2</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>Ischemia + zaprinast</td>
<td>5</td>
<td>270</td>
<td>0</td>
<td>2.1±0.3</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Ischemia + NO + zaprinast</td>
<td>5</td>
<td>270</td>
<td>0</td>
<td>2.1±0.3</td>
<td>1.4±0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, no. of lungs. $F_{O_2}$, inspired fraction of O$_2$; NO, nitric oxide. Microvascular pressure and hydrostatic challenge-induced lung weight gain were measured in continuously perfused control lungs, ischemic lungs, and ischemic lungs treated with NO, zaprinast, or a combination of both. During ischemia, lungs were ventilated with an O$_2$-free gas mixture, and a positive intravascular pressure was maintained. Significantly different ($P < 0.05$) from: *control lungs; †ischemic lungs.
DISCUSSION

In the present study, warm ischemia was employed in isolated lungs to provoke a massive leakage response as reflected by manyfold elevated $K_{fc}$ values and progressive severe pulmonary edema formation on reperfusion. Under these conditions, both inhalation of NO and administration of the PDE V inhibitor zaprinast from the onset of reperfusion exerted a moderate attenuation of the leakage response. The combined application of both agents did, however, exert a most impressive protective effect against the I/R-related hypopermeability, which was clearly beyond the potential of NO inhalation alone. In parallel, a severalfold increase in perfusate cGMP levels was noted on coadministration of NO and zaprinast, surpassing by far the levels of this cyclic nucleotide in response to either agent alone.

The experimental setup of warm ischemia and reperfusion in buffer-perfused rabbit lungs has been previously described (16, 35). During ischemia, two strategies of biophysical protection were employed in this model. First, lungs were ventilated with an O2-free gas mixture because ventilation-dependent dynamic mechanical forces were observed to attenuate ischemia-related lung injury (14, 35). Second, a positive intravascular pressure was maintained throughout the ischemic period, known to reduce the I/R-related leakage response by mechanisms hitherto not fully characterized in detail (3, 35).

During the initial reperfusion period, a transient and partially reversible increase in $P_{pa}$ was noted. However, this pressure rise was not relevant for the subsequent progressive leakage response for the following reasons: 1) when the postischemic $K_{fc}$ was determined 30 min after the onset of reperfusion, the elevated $P_{pa}$ values had returned to near baseline levels, and 2) the $P_{pc}$ levels were only marginally and very transiently affected by the I/R maneuvers and did not differ between the various groups at the time points at which the hydrostatic challenges were performed (in fact, when assessed 30 min after reperfusion, the mean $P_{pc}$ values in the ischemic lungs were even slightly lower than those of the control lungs). The transient and very moderate $P_{pc}$ elevation at the onset of reperfusion (~2 mmHg) may also not be considered as a mechanical “trigger” of the subsequent leakage response because $P_{pc}$ values reported to provoke stress failure, and thereby capillary hyperpermeability in the lung vasculature, range at values more than one order of magnitude higher (42). Therefore, the lung edema formation in the reperfusion period is largely independent of hydrostatic forces and must be ascribed to an impairment of pulmonary vascular barrier function. The fact that the capillary bed is the major site of lung fluid filtration as well as morphological examinations of lungs undergoing I/R injury (17) strongly supports the view that derangements of barrier properties of the lung microvasculature are largely responsible for the leakage in response to the I/R challenge.

Fig. 2. Impact of NO, zaprinast, or combined application of NO and zaprinast on the capillary filtration coefficient ($K_{fc}$) in reperfused rabbit lungs. At time 0, lungs were exposed to anoxic ischemia for 270 min. NO (10 ppm) and/or zaprinast (100 μM) was administered on reperfusion as indicated. Control lungs did not undergo ischemia but were continuously perfused in the absence of NO and zaprinast. Values are means ± SE; error bars not shown are within symbol. *P < 0.05 vs. zaprinast+NO. **P < 0.001 vs. zaprinast+NO. §P < 0.05 vs. control. §§P < 0.001 vs. control.

Fig. 3. Impact of NO, zaprinast, or combined application of NO and zaprinast on cGMP release into the buffer fluid in reperfused rabbit lungs. At time 0, lungs were exposed to anoxic ischemia for 270 min. NO (10 ppm) and/or zaprinast (100 μM) was administered on reperfusion as indicated. Values are means ± SE; error bars not shown are within symbol. *P < 0.05 vs. zaprinast+NO. *P < 0.001 vs. ischemia.
Against this background, the protective effects of NO and/or zaprinast may not be attributed to their vasodilatory properties, even when taking into consideration that both agents attenuated the $P_{pa}$ elevation occurring in the initial reperfusion period by $\sim$50%. This reasoning is further supported by the fact that 1) the $P_{pe}$ values assessed at the time points of hydrostatic challenge for measurement of $K_{fc}$ were nearly identical in all groups including those with NO or zaprinast treatment and 2) the reduction in the reperfusion-induced $P_{pa}$ elevation in the lungs with combined administration of NO and zaprinast did not surpass the suppressive effect of each single agent, whereas the impact of the combined treatment on the leakage response was markedly more prominent. Thus the protective effects of NO and zaprinast against $K_{fc}$ increase, and lung edema formation must be primarily ascribed to maintenance of capillary endothelial barrier function under conditions of I/R injury.

NO exerts several biological actions beyond its vasodilating properties. Many of these effects are mediated by activation of soluble guanylate cyclase and increased production of cGMP. In pulmonary artery monolayers, NO donors as well as dibutyryl cGMP (39) or 8-BrcGMP (43) blocked endothelial hyperpermeability. These protective effects may involve F-actin-related mechanisms (24), the activation of cGMP-dependent protein kinases (41), and the inhibition of Ca$^{2+}$ accumulation (10) in endothelial cells. Some direct impact of NO and zaprinast on the microvascular endothelial cell barrier properties to block the hyperpermeability on reperfusion may thus underlie the anti-edematous effect of these agents in the present study. Moreover, NO-cGMP may interfere with leukocyte endothelial interactions. Neutrophils are present in pulmonary capillaries even in buffer-perfused lungs, and previous morphometric analysis by this group (12) in fact demonstrated that the pool size of neutrophils sequestered in the microvasculature of the buffer-perfused rabbit lungs surpasses the pool size of this leukocyte population in the circulating blood volume of this species. These capillary neutrophils may thus contribute to the lung injury under conditions of I/R as previously suggested (37). NO has been demonstrated to inhibit the release of reactive oxygen species from neutrophils (8), and neutrophil adhesion-dependent alterations in microvascular permeability in the inflamed rat mesentery were blocked by NO and 8-BrcGMP (19). After I/R in mesenteric venules, protection by NO was related to a reduction in leukocyte-endothelium adhesion in rats (23) and cats (21). Moreover, in experimental lung injury, inhalation of NO has been shown to reduce neutrophil-mediated (13) or hydrogen peroxide-induced (32) microvascular leak. However, hitherto available data on the effects of NO inhalation in I/R-induced lung injury displayed conflicting results. Failure of protection or even enhancement of the leakage response was observed in rat lung transplantation models (11, 30). In contrast, inhaled NO has been demonstrated to reduce microvascular leakage in several other reperfusion models including isolated rabbit (16), rat (6, 27) and neonatal piglet (2) lungs. Moreover, intravascularly administered NO donors provided protection in reperfused isolated rat lungs (26) and enhanced the preservation of transplanted rat lungs (29). These beneficial effects of NO are obviously related to stimulation of soluble guanylate cyclase; NO-mediated reduction in the $K_{fc}$ was inhibited by a cGMP antagonist in rabbit lung I/R (6). Moreover, cGMP levels were found to decline in a rat model of lung transplantation (31), and the cGMP analog 8-BrcGMP as an additive to the preservation solution was found to improve pulmonary function in that study (31) as well as in reperfused rabbit lungs (20).

In the present study, a dose of 10 ppm NO was chosen for inhalation therapy because this dose was recently found to provide maximum protection against I/R injury in rabbit lungs, with both higher doses (>50 ppm) and lower doses (<1 ppm) being less effective (16). In addition, it was noted in that previous study that on prolongation of the ischemic time, even the optimum dose of NO inhalation lost its efficacy. It is in line with these observations that in the present study, with employment of the very long warm ischemic period of 4.5 h, limited protective capacity of NO inhalation against the leakage response to reperfusion was noted. Therefore, we attempted to enhance the efficacy of NO by inhibiting the breakdown of its second messenger cGMP by the selective PDE V inhibitor zaprinast to amplify the beneficial effect of the NO-cGMP axis but to avoid the disadvantageous impact of cGMP-unrelated toxic effects exerted by high NO doses. Zaprinast was previously shown to augment the vasodilatory impact of NO in models of pulmonary hypertension (18, 40). Zaprinast has also been found to reduce microvascular leak in guinea pig airways (34). The present study is the first to demonstrate that zaprinast and inhaled NO exert additive effects when coadministered to provide impressive protection against I/R-induced hyperpermeability and lung edema formation, with the efficacy of this combined regimen by far exceeding that of each single agent. Direct measurements of perfusate cGMP levels strongly support the view that the protective effect is, indeed, forwarded by the manyfold increased cGMP levels encountered under these conditions. As similarly discussed for the administration of NO alone, this beneficial effect of the NO-zaprinast combination in the present severe ischemic model may not be explained by the vasodilatory effects of this approach but must be attributed to some direct impact on the endothelial barrier damage encountered on reperfusion. The strong potency of coapplied zaprinast to enhance NO-induced cGMP accumulation and protection against hyperpermeability is in line with the finding that nearly all lung cGMP hydrolytic activity is attributable to the cGMP-specific PDE V targeted by zaprinast (9). The presence of PDE V has been demonstrated in the pulmonary vasculature of many species including human (15, 33).

In conclusion, coadministration of the cGMP-specific PDE inhibitor zaprinast strongly enhanced the beneficial effect of NO on endothelial barrier function in a rabbit lung model of severe I/R injury. The protective
effect of the combined regimen by far exceeded the maximum protection provided by each single agent and was associated with a manifold increased cGMP appearance in the lung perfusate. Detailed analysis of the hemodynamics showed that the NO-zaprinast effect is not forwarded via enhanced vasodilatory efficacy of this approach but is attributable to some direct impact on endothelial integrity on reperfusion. These data support the concept that PDE V inhibition is well suited to enhance the cGMP-related beneficial effects of inhaled NO in lung I/R while avoiding the disadvantageous impact of cGMP-unrelated toxic effects exerted by high NO doses.

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