INHALED NITRIC OXIDE FAILS TO CONFER THE PULMONARY PROTECTION PROVIDED BY DISTAL STIMULATION OF THE NITRIC OXIDE PATHWAY AT THE LEVEL OF CYCLIC GUANOSINE MONOPHOSPHATE

It has been suggested that inhaled nitric oxide gas may be beneficial after lung transplantation, because endogenous levels of pulmonary nitric oxide decline rapidly after reperfusion. However theoretical concerns remain about the formation of highly toxic oxidants during the quenching of nitric oxide by superoxide. To determine whether distal stimulation of the nitric oxide–cyclic guanosine monophosphate pathway at the level of cyclic guanosine monophosphate might confer the beneficial vascular effects of nitric oxide without its potential toxicities, we studied an orthotopic rat left lung transplant model. In this model, hemodynamic and survival measurements can be obtained independent of the native right lung. Lungs were preserved for 6 hours at 4° C in Euro-Collins solution alone (control, $n = 6$) or supplemented with the cyclic guanosine monophosphate analog, 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic guanosine monophosphate (cGMP, $n = 4$). In additional experiments in which lungs were preserved with Euro-Collins solution alone, inhaled nitric oxide was administered during reperfusion (NO, $n = 12$). Thirty minutes after transplantation and ligation of the native right pulmonary artery, pulmonary vascular resistance, arterial oxygenation, graft neutrophil infiltration (myeloperoxidase activity), and recipient survival were evaluated. Cyclic guanosine monophosphate decreased pulmonary vascular resistance (1.1 ± 0.2 vs 12.1 ± 6.3 mm Hg/ml/min, $p < 0.05$), improved oxygen tension (369 ± 56 vs 82.8 ± 48 mm Hg, $p < 0.05$), reduced myeloperoxidase activity (1.7 ± 0.3 vs 3.1 ± 0.9 Δ Abs 460 nm/min, $p < 0.05$), and improved recipient survival (100% vs 0%, $p < 0.005$) compared with Euro-Collins solution alone (control group). Animals receiving inhaled nitric oxide during reperfusion did not differ from control animals with respect to any of these parameters. These data suggest that distal stimulation of the nitric oxide–cyclic guanosine monophosphate pathway at the level of cyclic guanosine monophosphate has a protective effect that is not seen with inhaled nitric oxide in the immediate pulmonary reperfusion period. (J THORAC CARDIOVASC SURG 1995;110:1434-41)

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Current lung transplantation strategies are limited by the extreme vulnerability of the lungs to reperfusion injury after pulmonary preservation,

characterized by elevated pulmonary vascular resistance (PVR), poor gas exchange, and neutrophil infiltration.¹ Recent studies have demonstrated that

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maintaining endothelial function within transplanted organs is critical to successful preservation.²⁻⁶ Of the numerous factors influencing vascular function, nitric oxide (NO) functions as a key modulator of normal pulmonary vascular physiology, mediating vasodilation,^{7,8} preventing neutrophil adherence to the endothelium,^{9,10} maintaining endothelial barrier properties,¹¹ and inhibiting platelet aggregation.³ Inasmuch as endogenous pulmonary NO levels plummet during the first few minutes of reperfusion,⁴ some have suggested that inhaled NO may be beneficial after lung transplantation.^{12,13} Concerns remain, however, that inhaled NO may combine rapidly with superoxide generated during reperfusion to form highly toxic peroxynitrite and hydroxyl radicals,^{14,15} with potentially deleterious consequences for the newly reperfused pulmonary graft. Because NO exerts its beneficial effects on vascular function by stimulating soluble guanylate cyclase to increase cyclic guanosine monophosphate (cGMP) in effector cells,¹⁶ we hypothesized that distal stimulation of the NO/cGMP pathway at the level of cGMP might confer the beneficial vascular effects of NO without its potential toxicities.

Methods

Lung preservation and transplantation. Preservation solutions consisted of modified Euro-Collins solution (Baxter Healthcare Corp., Deerfield, Ill.; Na⁺, 10 mEq/L; K⁺, 115 mEq/L; Cl⁻, 15 mEq/L; HPO₄⁻, 85 mEq/L; H₂PO₄⁻, 15 mEq/L; HCO₃⁻, 10 mEq/L; modified by adding 10 ml of 10% magnesium sulfate and 50 ml of 50% glucose solution) or Euro-Collins solution supplemented with the membrane-permeable nonhydrolyzable cGMP analog, 8-(4-chlorophenylthio)-guanosine-3',5'-cGMP (8-pCPT-cGMP, BioLog Life Science Institute, La Jolla, Calif.). In inbred male Lewis rats (250 to 300 gm), the pulmonary artery was flushed with a 30 ml volume of 4° C preservation solution at a constant pressure of 20 mm Hg. The left lung was then harvested, a cuff was placed on each vascular stump, a cylinder was inserted into the bronchus, and the lung was submerged for 6 hours in 4° C preservation solution. Rats matched by gender, strain, and size were anesthetized, intubated, and their lungs ventilated with a rodent ventilator (Harvard Apparatus Co., South Natick, Mass.). Orthotopic left lung transplantation was performed through a left thoracotomy with a rapid cuff technique for all anastomoses, with warm ischemic times maintained below 10 minutes as described previously.^{4-6,17} The bronchial crossclamp was released first to allow gas distribution, after which the vascular clamps were released to reestablish blood flow. A snare was then passed around the right pulmonary artery, and Millar catheters (2F; Millar Instruments, Inc., Houston, Tex.) were introduced into the main pulmonary artery and the

left atrium. A flow probe (Transonic, Ithaca, N.Y.) was placed around the main pulmonary artery.

Experimental conditions. Three experimental conditions were tested: (1) Control: lungs were preserved in Euro-Collins solution alone and transplanted as described earlier; (2) NO: lungs were preserved in Euro-Collins solution alone, but NO inhalation (65 ppm, monitored by chemiluminescence) was begun immediately before reperfusion of the transplanted lung and continued during reperfusion; or (3) cGMP: 8-pCPT-cGMP (250 μmol/L) was added to the preservation solution. Because the carrier gas for NO administration was nitrogen, the gas mixture used for ventilation in all experiments was 70% oxygen and 30% nitrogen.

Measurement of lung graft function. On-line hemodynamic monitoring was accomplished with MacLab software and a Macintosh IIfx computer (Apple Computer, Cupertino, Calif.). Measured hemodynamic parameters included left atrial and pulmonary artery pressures (millimeters of mercury) and pulmonary artery flow (milliliters per minute). Arterial oxygen tension (PO₂, millimeters of mercury) was measured during inspiration of 70% oxygen and 30% nitrogen with a model ABL-2 gas analyzer (Radiometer A/S, Copenhagen, Denmark). PVRs were calculated as (mean pulmonary artery pressure – left atrial pressure)/mean pulmonary artery flow and expressed as millimeters of mercury per milliliter per minute. After baseline measurements, the native right pulmonary artery was ligated and serial measurements taken every 5 minutes until the animal was killed at 30 minutes (or until recipient death). Hemodynamic measurements were recorded at the final time at which the recipient was alive. Thirty minutes after ligation of the native right pulmonary artery, or at the time of recipient death, transplanted lungs were removed, rinsed briskly in physiologic saline solution, and snap frozen in liquid nitrogen until the time of myeloperoxidase assay, performed as described.¹⁸ Previous studies have shown 100% recipient survival with these techniques when lungs were harvested as described and immediately transplanted.¹⁷ In addition, although we chose a priori to use the 30-minute time after transplantation to evaluate grafts to study lung preservation in the immediate posttransplantation period, we have previously shown that well-preserved grafts can permit recipient survival well beyond the 30-minute observation period.⁶

Statistics. Data were evaluated by means of the Mann-Whitney U test or Fisher's exact test. Values are expressed as mean ± standard deviation, with differences considered statistically significant if $p < 0.05$.

Results

After 6 hours of preservation in Euro-Collins solution alone, grafts failed rapidly after ligation of the native pulmonary artery, with marked declines in pulmonary arterial flow and arterial oxygenation (Fig. 1, *A* and *D*). In sharp contrast, when 8-pCPT-cGMP was added to the preservation solution, hemodynamic (pulmonary artery flow) and functional

Fig. 1. Effect of inhaled NO or 8-pCPT-cGMP on pulmonary arterial flow (*PA flow*, ml/min, **A**, **B** and **C**) and arterial oxygenation (PO_2 , mm Hg, **D**, **E**, and **F**) after 6 hours of preservation at 4° C in Euro-Collins solution, followed by lung transplantation. The native (right) pulmonary artery was ligated at time 0. **A** and **D**, Control: Euro-Collins solution alone ($n = 6$), **B** and **E**, Inhaled NO: Euro-Collins solution alone, but the recipient's lungs were ventilated with inhaled NO gas (65 ppm, $n = 12$), **C** and **F**, 8-pCPT-cGMP: Euro-Collins solution supplemented with the membrane-permeable nonhydrolyzable cGMP analog, 8-(4-chlorophenylthio)-guanosine-3',5'-cGMP (250 $\mu\text{mol/L}$, $n = 4$). *PA*, Pulmonary artery.

(arterial PO_2) parameters were stabilized and recipients survived the 30-minute observation period (Fig. 1, **C** and **F**), whereas 8 of 12 recipients whose lungs were ventilated with NO gas during reperfusion showed declines in pulmonary arterial flow and arterial oxygenation similar to those observed in control animals (Fig. 1, **B** and **E**).

When pooled data are evaluated for all animals, 8-pCPT-cGMP added to the preservation solution significantly increased pulmonary artery flow (0.5 ± 0.3 vs 20.6 ± 2.1 ml/min, $p < 0.01$) (Fig. 2, **A**), decreased PVR (12.1 ± 6.3 vs 1.1 ± 0.2 mm Hg/ml per minute, $p < 0.05$) (Fig. 2, **B**), and improved arterial PO_2 (82.8 ± 48 vs 369 ± 56 mm Hg, $p <$

0.05) (Fig. 2, **C**), compared with values in the control group. Inhaled NO did not show similar beneficial effects (*PA flow*, 10.6 ± 16.0 ml/min; *PVR*, 6.5 ± 5.0 mm Hg/ml/min, PO_2 , 170 ± 174 mm Hg) (Fig. 2).

Because stimulation of the NO/cGMP pathway has important effects to inhibit neutrophil adherence to the reperfused coronary endothelium, and neutrophil infiltration has been implicated in the no-reflow phenomenon, we evaluated the effects of stimulation of this pathway on pulmonary graft neutrophil infiltration. Compared with Euro-Collins solution alone (control group), the cGMP analog 8-pCPT-cGMP added to the preservation solution was associated with a significant decline in lung

Fig. 2. Effects of inhaled NO or 8-pCPT-cGMP on lung preservation for transplantation. All lung transplants were performed after 6 hours of hypothermic preservation. Measurements were recorded at the final time at which the recipient was alive or at 30 minutes after ligation of the native right pulmonary artery. **A**, Pulmonary arterial flow (ml/min). **B**, Pulmonary vascular resistance (mm Hg/ml/min). **C**, Arterial oxygenation (mm Hg) ($n = 6$ for control, $n = 12$ for inhaled NO, and $n = 4$ for 8-pCPT-cGMP). Means $\pm$ standard deviation are shown.

myeloperoxidase activity (3.1 ± 0.9 vs 1.7 ± 0.3 Δ Abs 460 nm/min, respectively, $p < 0.05$) (Fig. 3). In contrast, inhaled NO gas did not alter neutrophil deposition (2.9 ± 0.6 Δ Abs 460 nm/min, $p = \text{NS}^*$ vs control group) (Fig. 3).

To determine whether the beneficial vascular effects of 8-pCPT-cGMP translated into improved recipient survival, we measured survival during the 30-minute observation period after ligation of the native pulmonary artery. These data showed survival to be significantly improved by supplementation of the preservation solution with the cGMP analog compared with controls (0% vs 100%, $p < 0.005$), but not by inhaled NO gas (33%, $p = \text{NS}$ vs controls) (Fig. 4).

Discussion

Lung transplantation is gaining increased acceptance as a therapeutic alternative for patients with end-stage lung disease.¹⁹ However the lungs are extremely vulnerable to ischemia-reperfusion injury, and current methods of preserving lungs for trans-

plantation often do not prevent the development of pulmonary dysfunction after transplantation.¹ Because endothelium-derived NO appears to play a critical role in maintaining vascular homeostasis after reperfusion²⁻⁶ and these levels plummet immediately on reperfusion of the transplanted lungs,⁴ we investigated whether a strategy to buttress NO levels at the time of reperfusion by administering NO by inhalation might confer beneficial vascular effects. There remains the theoretical concern, however, that the rapid interaction of exogenously administered NO with endogenous superoxide generated during pulmonary reperfusion may liberate far more toxic oxygen species, such as peroxynitrite and hydroxyl radical.^{14,15} This potentially deleterious interaction has been reported in cardiac reperfusion injury,²⁰ endothelial cells,¹⁵ and rat tubular hypoxia/reoxygenation injury.²¹ If this interaction were to occur to a significant extent in vivo in the setting of lung transplantation, we hypothesized that inhaled NO might be deleterious during early reperfusion and that distal stimulation of the NO/cGMP pathway with a cGMP analog might confer the beneficial vascular effects of NO while avoiding its potential toxicities.

* NS = Not significant.

Fig. 3. Effects of inhaled NO gas or 8-pCPT-cGMP on graft neutrophil accumulation. Myeloperoxidase activity (MPO; Δ Abs 460 nm/min) was used to quantify neutrophil deposition ($n = 6$ for control, $n = 12$ for inhaled NO, and $n = 4$ for 8-pCPT-cGMP). Means $\pm$ standard deviation are shown.

Fig. 4. Actuarial survival of the recipients ($n = 6$ for control, $n = 12$ for inhaled NO, and $n = 4$ for 8-pCPT-cGMP). *** $p < 0.005$ vs control at 30 minutes after ligation of the native pulmonary artery (PA).

The data presented here show that inhaled NO given during reperfusion had no beneficial pulmonary vascular effects compared with Euro-Collins solution alone. In contrast, the cGMP analog 8-pCPT-cGMP added to the preservation solution resulted in reduced PVR, improved arterial oxygenation, and attenuated graft neutrophil infiltration. The finding that 8-pCPT-cGMP enhances pulmo-

nary preservation is supported by the observation that nitroglycerin, which also stimulates guanylate cyclase to increase cGMP levels, is also beneficial after lung preservation.^{5,6}

These data are in contrast to several recent reports of inhaled NO given after lung transplantation, in which inhaled NO appeared to be beneficial. Although one report showed that inhaled NO re-

duced intrapulmonary shunting, reduced PVR, and improved oxygenation after human lung transplantation,¹³ NO was given from 12 hours to 10 days after transplantation, providing no assessment of the potential toxicities of inhaled NO during the critical early minutes of reperfusion. Another study, in which inhaled NO was given to dogs after lung transplantation, is difficult to assess, because prostaglandin E₁ was given to all animals.¹² Prostaglandin may itself attenuate neutrophil adhesion²² and therefore reduce the amount of superoxide generated by recruited neutrophils, obscuring potential toxicities related to exogenous NO administration. For the experiments described in the current article, we have elected not to use prostaglandin E₁ supplementation so as not to obscure the role of the cGMP second messenger cyclic nucleotide pathway. Preliminary data of ours suggest that stimulation of the cyclic adenosine monophosphate second messenger cyclic nucleotide pathway (which may result from prostaglandin E₁ supplementation²²) has overlapping effects with the cGMP pathway on the pulmonary vascular milieu.^{23, 24}

Inasmuch as our studies did not show a deleterious effect of inhaled NO compared with Euro-Collins solution (control group), this may reflect a precarious balance achieved between the toxic effects of NO and its beneficial vascular effects (vasodilation, inhibition of both neutrophil adhesion, and platelet aggregation). To circumvent the potential toxicities associated with the interaction of NO with superoxide, we used a membrane-permeable, non-hydrolyzable cGMP analog (8-pCPT-cGMP²⁵) to supplement the NO/cGMP pathway. In these experiments, addition of 8-pCPT-cGMP to the preservation solution resulted in a marked stabilization of pulmonary hemodynamics after transplantation, as well as attenuated graft neutrophil infiltration. Because neutrophil plugging has been implicated as an important cause of the no-reflow phenomenon,^{26, 27} attenuated neutrophil accumulation may have contributed further to the apparent reduction in PVR. In addition, reduced graft leukostasis may have reduced the toxicity of the postreperfusion vascular milieu, because neutrophils release reactive oxygen intermediates, proteases, and other toxic compounds.²⁸ In further support of the potential therapeutic utility of a cGMP analog, inhalation of the cGMP analog 8-bromo-cGMP has been shown to selectively lower PVR in a porcine model of pulmonary hypertension.²⁹ Together, these data suggest that targeted delivery of a cGMP analog either by

inhalation or by intrapulmonary instillation (as by pulmonary artery flushing during preservation) can have beneficial pulmonary vascular effects.

Taken together, these data suggest that stimulation of the NO/cGMP pathway at the level of cGMP, by supplementing the preservation solution with a cGMP analog, has a protective effect on an orthotopic rat left lung transplant model that is not seen with inhaled NO given in the immediate reperfusion period. These data contribute to the growing body of evidence indicating the important role of the graft vasculature in pulmonary preservation.

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Discussion

Dr. Steven J. Mentzer (*Boston, Mass.*). There is a tendency in the medical literature to refer to ischemia and reperfusion as a single event. They are in fact two events, cellular hypoxia followed by reoxygenation. Many of the discordant findings in the field of NO and organ preservation are a result of cross-wiring these two events. An important aspect of the work presented by Dr. Naka is that it begins to differentiate the effects of cellular hypoxia from the injury caused by reoxygenation. I have two comments regarding cellular hypoxia and the lung. First, a common misconception is that a hypoxic microenvironment uniformly inhibits cellular functions. We now know that the opposite can be true. Hypoxia can be a potent stimulus for both gene transcription and cellular metabolism. For example, relative hypoxia can trigger endothelial cells to transcribe the potent vasoconstrictors endothelin-1 and platelet-derived growth factor, or PDGF. In most physiologic circumstances, such as organ preservation, the hypoxia-induced production of vasoconstrictors can result in a deleterious imbalance in vasoregulators.

By including a cGMP analog in the lung perfusate, these investigators have anticipated this imbalance and have blunted the hypoxic responses of the lung. This type of preemptive metabolic manipulation will doubtless play an increasingly important role in treatment of ischemia in the future.

Second, a word of caution. Inhaled NO facilitates ventilation-perfusion matching precisely because it is inhaled. By definition, vasodilation induced by inhaled NO occurs in areas that are being ventilated. In contrast, the distal stimulation of the NO pathway described Dr. Naka with soluble mediators is independent of ventilation. Soluble mediators that stimulate the distal NO pathway should be used cautiously to avoid unanticipated ventilation-perfusion mismatching.

Dr. Naka. Thank you for your excellent comments, Dr. Mentzer. We are also considering the properties of ischemic endothelium.

You mentioned about the data reported recently in which inhaled NO given during transplantation appeared to be beneficial. In the one report of human lung transplantation, inhaled NO was not given during the critical early minutes of reperfusion, providing no assessment of

the potential toxicity of inhaled NO given during this period. In another report of canine lung transplantation, prostaglandin E₁, which may itself attenuate neutrophil infiltration and reduce superoxide generation from neutrophils, was given to all animals in addition to NO, potentially obscuring the effects of exogenous inhaled NO.

Dr. Shaf Keshavjee (*Toronto, Ontario, Canada*). Did your cGMP analog have any effects on the systemic circulation in your model.

Dr. Naka. Although we did not evaluate the systemic effects of the cGMP analog in our model, all animals

receiving the cGMP analog survived the 30-minute observation period after transplantation, whereas all of control grafts failed. If an adverse effect existed, it did not affect graft function or recipient survival.

Dr. John H. Kennedy (*Cambridge, England*). Hypocarbapria apprizes vasoconstriction in cerebral vessels as some other systems. Did you measure carbon dioxide tension as you measured PO₂?

Dr. Naka. We also measured carbon dioxide tensions in all experiments and they did not show any significant differences between the groups.