LASTING BENEFICIAL EFFECT OF SHORT-TERM INHALED NITRIC OXIDE ON GRAFT FUNCTION AFTER LUNG TRANSPLANTATION

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The combination of ischemia and reperfusion after lung transplantation is characterized by endothelial damage, neutrophil sequestration, and decreased release of endothelial nitric oxide. Because nitric oxide has been shown to selectively dilate the pulmonary vasculature, abrogate neutrophil adherence, and restore endothelial dysfunction, we hypothesized that inhaled nitric oxide given for 4 hours during initial reperfusion might attenuate reperfusion injury in a porcine model of left single-lung transplantation. We tested hemodynamic and gas exchange data, lung neutrophil sequestration, and pulmonary artery endothelial dysfunction after 4 and 24 hours of reperfusion in 12 pigs randomly assigned to nitric oxide and control groups. Harvested lungs were preserved in normal saline solution for 24 hours at 4° C. During transplantation, inflatable cuffs were placed around each pulmonary artery to allow separate evaluation of each lung by occluding flow. Compared with the transplanted lungs in the control group, transplanted lungs in pigs treated with inhaled nitric oxide significantly improved gas exchange, pulmonary vascular resistance, shunt fraction, and oxygen delivery at 4 and 24 hours after reperfusion. Neutrophil sequestration, as measured by the neutrophil-specific enzyme myeloperoxidase and the alveolar leukocyte count per light microscopic field, was significantly lower at 24 hours after reperfusion in the transplanted lungs of the nitric oxide group. The nitric oxide-treated native right lungs exhibited significantly reduced increase in neutrophil accumulation compared with that in control native right lungs. After 24 hours of reperfusion, endothelium-dependent relaxation to acetylcholine was similarly and severely altered in both groups. We conclude that short-term inhaled nitric oxide given during the first 4 hours of reperfusion after lung transplantation significantly attenuates reperfusion injury, improving graft function as long as 24 hours after operation. This effect is probably mediated by a decrease in neutrophil sequestration. A protective effect on the contralateral lung was also observed. Inhaled nitric oxide may be a suitable agent when an acute reperfusion phenomenon is anticipated. (*J Thorac Cardiovasc Surg* 1996;112:590-8)

Early death after lung transplantation is mainly a function of ischemia-reperfusion (IR) injury, which occurs in about 20% of recipients.¹ This complex lung injury is characterized by increased pulmonary capillary permeability, pulmonary hypertension, and peripheral leukopenia.²⁻⁴ Endothelial

damage, the hallmark of this phenomenon,⁵ appears to be the consequence of pulmonary sequestration and activation of polymorphonuclear neutrophils (PMNs).^{2,3,6} After sudden reoxygenation, toxic waste products combine to produce non-neutrophil-derived oxygen free radicals.^{7,8} This leads to endo-

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thelial adhesion and activation of PMNs.⁹ The release of inflammatory mediators further enhances this process.¹⁰ In turn, activated PMNs release reactive oxygen species and cytotoxic enzymes.¹¹ Pulmonary sequestration and activation of PMNs thus appear to play a key role in the pathogenesis of IR injury.⁸

Inhaled nitric oxide (NO) is being increasingly used as a potent selective pulmonary vasodilator,¹² effective in reducing pulmonary artery (PA) pressure in pulmonary hypertension after lung transplantation.¹³ Endothelial cell dysfunction in IR injury is manifested by a loss of NO-dependent vasodilation⁸ and correlates well with increased pulmonary vascular resistances after lung transplantation.¹⁴ A decreased release of endothelial NO is observed after reperfusion of transplanted rat lungs.¹⁵ NO has also been shown to decrease PMN adherence to reperfused endothelium⁸ and to maintain endothelial barrier properties.¹⁶ Previous observations by our group have shown that the reduction of pulmonary endothelium-dependent relaxation seen in IR injury was restored when the NO precursor L-arginine was given.¹⁷ In addition, inhaled NO was shown to prevent endothelial dysfunction and PMN accumulation in the isolated piglet lung.¹⁸ NO has also been shown, however, to potentiate the harmful effects of IR in some models.¹⁹ We therefore tried to confirm the beneficial effects of 4 hours of inhaled NO on pulmonary graft function and PMN sequestration in a large animal model after 4 and 24 hours of reperfusion. We tested whether NO (1) prevents an increase in pulmonary vascular resistance, (2) improves transalveolar gas exchange, (3) decreases pulmonary PMN sequestration, and (4) attenuates endothelial dysfunction 24 hours after lung transplantation.

Material and methods

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Left lung allotransplantation in pigs. Young pigs (Large White, $n = 24$, 25 to 30 weeks of age, 22 to 26 kg) were used. They were sedated with intramuscular ketamine (100 mg/kg), anesthetized with intravenous pentobarbital (10 mg/kg) followed by a continuous intravenous infusion of $0.1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, and paralyzed with pancuronium (0.3 mg/kg). After endotracheal intubation, the animals were ventilated with intermittent positive-

pressure ventilation (MMS 107 ventilator, Chelles, France) at a tidal volume of 15 ml/kg, with a respiratory rate of 18 cycles/min and a fraction of inspired oxygen (FiO_2) of 0.5. Body temperature was maintained at 37°C by means of a heating blanket.

Pigs were randomly allocated to the NO or control group ($n = 6$ in each group). Mean body weights of donor animals were 24.1 ± 0.7 kg in the control group and 23.5 ± 0.9 kg in the NO group (p not significant). In the recipient animals, mean body weights were 24.7 ± 0.8 kg and 24.0 ± 0.9 kg in control and NO groups, respectively (p not significant).

Donor operation. A median sternotomy was performed and the heart-lung block was dissected. After systemic heparinization (500 U/kg), a cannula was inserted into the main PA. The PA was flushed with 50 ml/kg cold (4°C) leukocyte-depleted (leukocyte filters; Pall Corp., Biomedical Products Div., Glen Cove, N.Y.) Wallworks blood-based solution. Ventilation was continued throughout the procedure. The trachea was clamped with the lungs inflated. The heart-lung block was harvested from right to left, to minimize trauma to the left lung. The left lung was then dissected from the heart-lung block with a large cuff of left atrial tissue and placed in a basin containing 0.9% cold saline solution and stored at 4°C for 24 hours.

Recipient operation. The weight-matched pigs were sedated and anesthetized with the same procedure used for the donor animals. A 750 mg dose of cefamandole was given intravenously before incision. A left thoracotomy was performed through the fifth intercostal space, and the pericardium was opened. The hilum was dissected and an inflatable cuff (Phymep, Paris, France) was placed around the proximal right PA. Care was taken to place the cuff above the most proximal branch. The left pulmonary veins, atrium, PA and main bronchus were dissected and clamped, and the left lung was removed. The bronchial anastomosis was performed first with continuous 4-0 Prolene suture (Ethicon, Inc., Somerville, N.J.). The left atrium was then anastomosed by performing a horizontal, continuous everting mattress suture with 6-0 Prolene suture. The PA anastomosis was performed with two continuous 6-0 Prolene sutures. A 120 mg dose of methylprednisolone was administered intravenously. After lung inflation, pulmonary circulation was restored. Another inflatable cuff was then placed around the left PA, and the injection ports connected to each cuff were exteriorized through the end of the thoracotomy. The lungs were ventilated with 100% FiO_2 and 3 cm H_2O positive end-expiratory pressure with the previously mentioned volumes. A chest tube was inserted, and the chest was closed. The chest tube was connected to a water-sealed drainage device with 20 cm H_2O suction, and the animal was placed in the prone position.

Clinical measurements. A Swan-Ganz flow-directed thermal-dilution catheter (Baxter Healthcare Corp., Edwards Division, Irvine, Calif.) was inserted through an external jugular vein cutdown and placed in the main PA with fluoroscopic guidance. A carotid arterial line was also placed. Both catheters were then connected to Bentley 800 transducers (Bentley Laboratories, Uden, Holland) and systemic arterial pressures and pulmonary arterial pressures were continuously monitored, with the zero

reference placed at midchest level. Pressures and electrocardiogram were recorded on a Gould Brush 2800 recorder (Gould, Inc., Oxnard, Calif.). Cardiac output (CO) was measured in triplicate by thermodilution with an Edwards 9502 computer and injection of 5 ml ice-cold 5% dextrose solution into the proximal port of the Swan-Ganz catheter. Pulmonary compliance was measured by the ratio of peak inspiratory pressure to tidal volume. Blood gas values (arterial and mixed venous) were measured by a blood gas analyzer (ABL 113; Radiometer America, Inc., Medical Div., Westlake, Ohio) at 37° C but were corrected for each animal's blood temperature.

Formulas used were as follows: Pulmonary vascular resistance (PVR) = Mean PA pressure/CO $\times$ 80 (dyne $\cdot$ sec $\cdot$ cm⁻⁵); Arterial oxygen content = Arterial saturation $\times$ 1.34 $\times$ hemoglobin (Hb) + 0.0003 $\times$ Arterial oxygen tension (Pao₂ in ml/dl); venous oxygen content = Venous saturation $\times$ 1.34 $\times$ Hb + 0.0003 $\times$ Venous oxygen tension (in ml/dl); Alveolar-arterial gradient = [Fio₂ $\times$ (760 - 47)] - arterial carbon dioxide tension - Pao₂ (in mm Hg); Shunt fraction at 100% Fio₂ = (Alveolar oxygen content - Arterial oxygen content)/(Alveolar oxygen content - Venous oxygen content) (in %).

Protocol. NO, supplied as a mixture of NO (300 ppm) in nitrogen, was added to the breathing circuit to produce an inspired concentration of 30 ppm. The concentrations of NO and nitrogen dioxide were assessed by a chemoluminescence method (NO $\times$ 2000, Seres, Aix en Provence, France) by sampling gas in the inspiratory limb of the circuit 2 cm proximal to the endotracheal tube and distally at the end of the endotracheal tube. Ventilation with NO was started 5 minutes before reperfusion and continued for 4 hours. The control group received the same concentration of nitrogen in the inspired gas through the same period. In both groups, lung function was assessed 4 and 24 hours after reperfusion. This assessment consisted of measurement of arterial and mixed-venous gas values, pulmonary compliance, and pulmonary and systemic hemodynamic values at an Fio₂ of 1.0 under the following three conditions: both lungs perfused, native right lung only perfused, and transplanted left lung only perfused. Each PA was left occluded for 10 minutes before measurement, and flow was restored for 10 minutes for equilibration before proceeding. Positive end-expiratory pressure remained the same. At the end of the 4-hour period, the animal was awakened and extubated. Twenty-four hours after reperfusion, the animal was anesthetized again and intubated. A median sternotomy was performed, and complete hemodynamic and gas exchange assessment was done under the same conditions as at 4 hours. Instead of using the cuffs, direct clamping of each PA was done. At autopsy, all anastomoses were inspected for stenosis.

Wet-dry lung weight (W/D) ratio. The native and transplanted lungs were excised and weighed for determination of the final wet lung weight. They were then dried in an oven at 60° C and weighed daily for 30 days or until the dry weight was stable for longer than 5 days, to allow determination of W/D ratio.

Leukocyte counts. Absolute and differential leukocyte counts were determined with an Argos counter (ABX, Paris, France). The automated counts were routinely

verified by manual countings. The counts, expressed as cells per milliliter, were used to calculate the percentage of PMNs' decrease as the ratio of difference in the PMN count before reperfusion and at 4 or 24 hours after reperfusion to the PMN count before perfusion.

Alveolar leukocytes (histology). At the end of the experiment, large specimens were taken from the upper and lower lobe of each lung and fixed by immersion in 10% neutral buffered formalin. After staining with hematoxylin-eosin, all biopsy samples were examined in a blinded fashion. Leukocyte counts were performed with a 40 $\times$ ocular and a 10 $\times$ eyepiece reticule to examine 10 consecutive fields containing only alveoli.

Myeloperoxidase (MPO) activity. At 24 hours, another lung tissue specimen was sampled and snap frozen in liquid nitrogen. For determination of baseline values, a specimen was taken from the freshly harvested donor right lung. MPO, a marker enzyme specific to PMNs, was used as an indirect measure of tissue neutrophil infiltration. The method described by Mullane, Kraemer, and Smith²⁰ was used. Lung tissue was homogenized in 10% weight/volume solution of hexadecyltrimethyl ammonium bromide buffer (0.5% hexadecyltrimethyl ammonium bromide in 50 mmol/L phosphate buffer at pH 6.0) with a Polytron homogenizer (Kinematica, Luzern, Switzerland). The homogenate was sonicated on ice for 15 seconds, frozen at -70° C and thawed three times, then centrifuged at 40,000g for 15 minutes. Supernatant was assayed spectrophotometrically for MPO activity. A 20 μ l portion of the supernatant was combined with 12 μ l 25 mmol/L hydrogen peroxide, 30 μ l 40 mmol/L *o*-dianisidine hydrochloride, and 1.938 ml 50 mmol/L phosphate buffer (pH 6.0). The change in absorbance was measured at 460 nm on a Beckman spectrometer (model 25 spectrometer; Beckman Instruments, Fullerton, Calif.). One unit of MPO activity was defined as the activity degrading 1 μ mol peroxide per minute at 25° C.

Isolated PA ring studies. After autopsy at 24 hours, lung specimens were harvested for isolated PA ring studies.¹⁸ Left and right intrapulmonary artery segments were dissected out, cleaned, cut into rings 3 to 4 mm in length (1 to 2 mm external diameter), and placed in warmed Krebs-Henseleit buffer composed of 118.3 mmol/L sodium chloride, 4.7 mmol/L potassium chloride, 2.5 mmol/L calcium chloride dihydrate, 1.2 mmol/L potassium phosphate, 1.2 mmol/L magnesium sulfate heptahydrate, 25 mmol/L sodium bicarbonate, 0.03 mmol/L ethylenediaminetetraacetic acid, and 11.1 mmol/L glucose. Three to four rings were obtained from each animal. For baseline values, rings were also obtained from the freshly harvested right donor lungs.

The rings were then mounted on stainless-steel hooks, suspended in 10 ml tissue baths, and connected to force-displacement transducers (LB-5; Rikadenki Electronics, Freiburg, Germany) to record changes in force with a strip-chart recorder (LR 4210; Yokogawa Electric Corp., Tokyo, Japan). The baths were filled with 10 ml Krebs-Henseleit buffer and aerated at 37° C with a gas mixture of 95% oxygen and 5% carbon dioxide. PA rings were initially stretched to produce a preload of 2g force and equilibrated for 60 to 90 minutes. A preload of 2g has been proved to be optimal for porcine PA rings.¹⁸ During

Fig. 1. Pao₂ after 4 and 24 hours of reperfusion with ventilation at Fio₂ of 1 and blood flow to the native right lung (NL) or to the transplanted left lung (TL) alone. † indicates $p < 0.001$ versus TL at 4 hours in the control group; * indicates $p < 0.05$ versus TL at 24 hours in the control group.

this period, the Krebs-Henseleit buffer in the tissue baths was replaced every 10 minutes. After incubation with indomethacin (10^{-5} mmol/L) for 60 minutes (to exclude any interaction with prostanoids), a concentration-response curve to phenylephrine (10^{-9} to 3×10^{-4} mmol/L final concentration) was obtained. The rings were then washed, and the developed force was allowed to return to baseline. The rings were precontracted with phenylephrine at the dose needed to achieve 50% of maximal contraction (EC₅₀). Once a stable contraction was obtained, acetylcholine hydrochloride (10^{-9} to 10^{-4} mmol/L final concentration) was added to the bath to assess changes in endothelium-dependent relaxation. These rings were washed again and allowed to equilibrate to baseline. The procedure was repeated with sodium nitroprussate, an endothelium-independent vasodilator (10^{-5} mmol/L). In addition to the change in force (in grams), responses were assessed by determining the following: (1) the EC₅₀ and (2) the maximal responses to phenylephrine and acetylcholine (E_{max}), which were extrapolated from the individual concentration-effect curves. Relaxation was expressed as the percentage decrease in tension of phenylephrine-elicited constriction. Acetylcholine hydrochloride and indomethacin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Phenylephrine and sodium nitroprussate were provided by Boehringer Ingelheim (Paris, France) and Hoffman-LaRoche & Co AG (Basel, Switzerland), respectively. All chemicals were freshly prepared the day of the experiment.

Statistical analysis. All values are expressed as the mean ($\pm$ standard error of the mean). Statistical analysis was performed with the paired Student's *t* test, two-way

Fig. 2. PVR after 4 and 24 hours of reperfusion, ventilation with Fio₂ of 1, and blood flow to the native right lung (NL) or to the transplanted left lung (TL) alone. † indicates $p < 0.001$ versus TL at 4 hours in the control group; * indicates $p < 0.01$ versus TL at 24 hours in the control group.

analysis of variance for repeated measurements, and the Newman-Keuls test as a post hoc test when the overall *F* value was significant. Values of *p* lower than 0.05 were considered significant.

Results

Both groups were comparable with respect to animal weight, duration of cold ischemia (24.0 ± 0.3 vs 24.3 ± 0.2 hours in the control and NO groups, respectively), warm ischemia (51 ± 8 vs 48 ± 12 minutes in the control and NO groups, respectively), and preoperative blood gases and pulmonary arterial pressures. Preoperative and postoperative Hb values and platelet counts were similar in the two groups. At autopsy, one pig was found to have thrombosis of the inferior left pulmonary vein and was excluded from the study.

Effect of inhaled NO on lung function and hemodynamic parameters. Ischemia and subsequent reperfusion resulted in marked deterioration of the overall function of the transplanted lung. With blood flow directed to the transplanted lung alone and an Fio₂ of 1, the Pao₂ (Fig. 1) was significantly greater in the NO group after 4 and 24 hours of reperfusion. Similarly, both PVR (Fig. 2) and shunt fraction (Table I) were significantly lower in the NO group after 4 and 24 hours of reperfusion. Inhaled

Table I. Summary of hemodynamic and gas-exchange measurements at 4 and 24 hours after left lung transplantation, ventilation with an inspiratory fraction of 1, with or without treatment with inhaled nitric oxide during the first 4 hours of reperfusion and with blood flow to both lungs, native right lung, or transplanted lung alone

	After 4 hours of reperfusion						After 24 hours of reperfusion					
	Control			Inhaled nitric oxide			Control			Inhaled nitric oxide		
	2L	NL	TL	2L	NL	TL	2L	NL	TL	2L	NL	TL
PaO ₂ (mm Hg)	359 ± 47	435 ± 33	96 ± 22	463 ± 23	463 ± 16	405 ± 31*	283 ± 51	359 ± 88	100 ± 31	367 ± 35	414 ± 32	233 ± 41†
Paco ₂ (mm Hg)	26 ± 3	26 ± 2	46 ± 5	30 ± 3	33 ± 8	38 ± 8	36 ± 13	32 ± 9	47 ± 8	23 ± 2	17 ± 3	25 ± 6†
Arterial oxygen saturation (%)	99 ± 0	99 ± 0	92 ± 3	99 ± 0	99 ± 0	99 ± 0‡	98 ± 1	98 ± 0	78 ± 16	99 ± 0	99 ± 0	97 ± 1.4†
Venous oxygen saturation (%)	62 ± 6	67 ± 5	27 ± 5	74 ± 7	71 ± 5	61 ± 10‡	48 ± 4	57 ± 3	26 ± 11	55 ± 7	52 ± 7	48 ± 9
MPAP (mm Hg)	22 ± 3	22 ± 3	43 ± 5	14 ± 3	15 ± 4	20 ± 4*	26 ± 4	26 ± 5	49 ± 8	16 ± 2	16 ± 2	24 ± 3*
CaO ₂ (ml/dl)	12 ± 1	12 ± 1	10 ± 1	14 ± 1	13 ± 1	13 ± 1‡	12 ± 1	13 ± 1	9 ± 2	13 ± 1	13 ± 1	12 ± 1†
Shunt fraction (%)	17 ± 1	16 ± 1	39 ± 8	15 ± 2	16 ± 2	20 ± 4‡	22 ± 2	18 ± 1	52 ± 18	21 ± 4	23 ± 4	28 ± 9†

2L, Both lungs; NL, native lung; TL, transplanted lung; Paco₂, arterial carbon dioxide tension; MPAP, mean PA pressure; CaO₂, arterial oxygen content. Values are mean ± standard error of the mean.

* $p < 0.001$ versus TL in control group.

† $p < 0.05$ versus TL in control group.

‡ $p < 0.01$ versus TL in control group.

NO did not affect other hemodynamic parameters, such as heart rate, systemic pressure, or CO. There was a tendency toward a preserved CO in the presence of NO (2.1 ± 0.4 vs 3.2 ± 0.4 L/min at 4 hours, and 1.6 ± 0.5 versus 2.8 ± 0.3 L/min at 24 hours); although not statistically significant, it translated into a significantly improved oxygen delivery value for the transplanted lung at 4 hours (228 ± 43 vs 452 ± 74 ml/min, $p < 0.05$).

The W/D ratios for the transplanted lungs were comparable in the two groups. W/D ratio was 5.1 ± 0.2 in the control group and 4.9 ± 0.1 in the NO group.

Effect of inhaled NO on pulmonary neutrophil sequestration. Inhaled NO was associated with a significant reduction in graft neutrophil sequestration (Fig. 3). In comparison with NO-treated transplanted left lungs, control transplanted left lungs had a significantly higher concentration of neutrophils within their tissue. Furthermore, the control right lung exhibited a statistically significant increase in MPO value compared with the baseline value, whereas the NO-treated right native lungs did not exhibit a significant increase from baseline (Fig. 3). This points to an injury-preventive effect of NO on the native lungs. An indirect parameter of reduced neutrophil sequestration, the absolute neutrophil count increased significantly in the NO group; such an increase did not occur in the control group,

presumably because of neutrophil sequestration in the graft (Fig. 4). Buttressing these findings, histologic examination of the transplanted lung showed massive leukocyte sequestration in the control group (170 ± 48 leukocytes per 10 fields) compared with the NO group (40 ± 8 leukocytes per 10 fields, $p < 0.05$). In the native lungs, leukocyte counts per 10 fields were similar in both groups.

Effect of inhaled NO on endothelium-dependent relaxation of PA rings. After 24 hours of reperfusion, the relaxant responses to acetylcholine of PA rings taken from transplanted and native lungs in both groups (as demonstrated by $E_{\max}$ and EC_{50}) were significantly reduced compared with baseline values obtained from PA rings derived from freshly collected vessels from the flushed right lung of the donor ($E_{\max}$ $46.8\% \pm 12.7\%$; EC_{50} $1.4 \cdot 10^{-7} \pm 0.5 \times 10^{-7}$ mol/L; $p < 0.05$ versus either group). For the transplanted lungs, there were no significant differences between the NO and control groups ($E_{\max}$: $23.2\% \pm 6.06\%$ and $15.8\% \pm 9.8\%$; EC_{50} $1.7 \times 10^{-6} \pm 0.7 \cdot 10^{-6}$ mol/L and $2.7 \times 10^{-6} \pm 1.3 \times 10^{-6}$ mol/L for the NO and control groups, respectively; Fig. 5). Relaxation responses were not significantly different from those of the reperfused native right lungs in either group. Endothelium-independent relaxation induced by sodium nitroprusside remained unaltered (ranging from 90% to 100%) and equal in both groups.

Fig. 3. Lung MPO activity at baseline (freshly harvested lungs) or from native lungs (NL) and transplanted lungs (TL) after 24 hours of reperfusion. * indicates $p < 0.01$ versus TL in the control group; † indicates $p < 0.001$ TL versus baseline within NO group, NL versus baseline, and TL versus NL within control group; § indicates $p < 0.0001$ TL versus baseline in control group.

Discussion

This study shows that inhaled NO applied for a period of 4 hours after reperfusion attenuates IR injury, resulting in improved graft function even after withdrawal of NO. This effect is maintained as long as 24 hours after reperfusion (endpoint of the study). To characterize the mechanisms involved in this protective effect, two key parameters of IR injury were quantified: pulmonary PMN sequestration and pulmonary endothelial function. We documented a significant decrease in PMN sequestration in the graft and a nonsignificant reduction in endothelial dysfunction.

The in vivo model used allowed a gas-exchange study, one of the most sensitive indicators of transplanted lung function.⁶ Direct comparison of the transplanted and native lungs was made possible through the use of the inflatable cuffs placed around each PA. This is a well described and reproducible model.^{21, 22} Performing a sternotomy at 24 hours allowed us to clamp each PA under direct vision, thus excluding any technical bias stemming from malfunctioning or leaking cuffs. The animals were kept to a rigorous functional standard; all were extubated after 6 to 7 hours and reintubated the next day. This contrasts with acute studies, in which animals are kept intubated until the end of the study period and then are killed. Furthermore, a 24-hour graft cold ischemia period has been previously shown to produce significant IR injury.⁴ Indeed, severe functional alterations and massive pulmonary PMN sequestration were consistently observed in

Fig. 4. Percentage of peripheral blood PMN increase after 4 and 24 hours of reperfusion in the control and NO groups. * indicates $p < 0.05$ versus control group at 4 hours.

the transplanted lungs of the control group but not those of the NO group. The blood used in the Wallwork's solution used for flushing was filtered to eliminate leukocytes. This was done to avoid PMN sequestration in the lung during the ischemic preservation, which could interfere with PMN measurements.

Prevention of IR injury after lung transplantation has been studied in different models, including the use of free-radical scavengers in the flush solution,²³ leukocyte-depleted blood,² cytokine inhibition with pentoxifylline,²² or NO donors.^{16, 17} These were all overwhelmingly acute studies, with variables measured within a 4- to 6-hour period after reperfusion. Leukocyte sequestration has also been shown to be largely diminished in these models, further pointing to the central role of PMNs in IR injury. The clinical and practical applicability of some of these techniques remain questionable, however, in immunosuppressed patients. Our choice to use inhaled NO rather than other NO donors was based on the fact that gaseous NO can be delivered directly to the pulmonary circulation by inhalation. Its short half-life is the result of rapid inactivation by Hb, which limits its systemic effects and restricts its activity to pulmonary tissues.⁸ To date, only a few reports have been published on inhaled NO in the treatment of acute reperfusion injury after lung transplantation.^{13, 24} Our results seem to indicate that inhaled NO has a preventive effect on IR injury and support selective pulmonary vasodilatory properties of in-

Fig. 5. Acetylcholine-induced endothelium-dependent relaxation (percentage decrease in tension of phenylephrine-elicited contraction) of PA rings derived from left transplanted lungs after ischemia and 24 hours of reperfusion for the NO (closed circles) and control (open circles) groups and for the baseline (freshly harvested lungs; triangles). * indicates $p < 0.05$ baseline versus NO and control group.

haled NO; no systemic side effects were noted. The largest human experience with NO is in the treatment of acute respiratory distress syndrome, and many studies have documented its low toxic side effect profile.²⁵ Furthermore, inhaled NO has also been found to be protective against oxidant-induced lung injury, a model related to IR injury.²⁶

IR injury is an explosive phenomenon that occurs within 2.5 minutes of reperfusion in animal models,²⁷ and the first hour of reperfusion is the period of higher risk of PMN-induced lung injury.²⁸ Evidence is accumulating that the bulk of the cellular injury and endothelial insult does not occur during ischemic preservation, a period of relative metabolic inertia, but during the reperfusion period.⁸ Consequently, our hypothesis was that NO would be maximally effective if applied during reperfusion, rather than during the preservation period.^{8,29} Nevertheless, the L-arginine/NO pathway has also been shown to be beneficial when activated during the period of preservation, as shown in a recent study demonstrating an enhanced preservation of pulmonary grafts after supplementation of the preservation solution with NO donors.^{16,30}

The lung seems to be particularly susceptible to

mediators released during IR injury.^{3,31} This may be related to the fact that the pulmonary endothelium represents the largest vascular bed in the body. Reperfusion of one ischemic lung results in significant contralateral damage.^{3,31} Our results support that notion because function of the control native right lungs was worse after reperfusion of the left transplanted lung in our model, although not significantly worse, than in the NO-treated native lungs. In addition, MPO values were significantly elevated in the control native lung compared with baseline values, confirming Bishop, Chi, and Cheney's findings³ that unilateral lung reperfusion results in PMN accumulation in the contralateral lung. Such an increase was not seen in the NO-treated native lungs. NO thus seems to inhibit PMN sequestration in the nontransplanted lung as well, conferring an obvious protection.

The marked improvement in transalveolar gas exchange and oxygen content seen in the NO-group could be the result of improved ventilation-perfusion matching because NO has the singular property of preferentially dilating vascular segments located in ventilated areas.²⁵ In contrast to the findings by Hillman and colleagues³² obtained in an adult respiratory distress syndrome model, in which Pao_2 values returned to baseline and rebound pulmonary hypertension was seen when NO was precipitously discontinued, we found that the beneficial effect exerted by NO could still be documented 24 hours later. These data support a different mechanism of action than the pure vasodilative effect of NO. Indeed, graft sequestration of PMNs was markedly inhibited by NO. This is probably a result of the well-described ability of NO to abrogate PMN adherence and diapedesis through the reperfused endothelium,^{8,29} protecting endothelial cells and the subjacent pneumocytes from PMN-generated oxygen-derived free radicals. The mechanism by which inhaled NO suppresses PMN adhesion may be related to a downward regulation of adhesion molecule expression by NO.³³ Inhibition of CD11-CD18 PMN adhesion molecules by monoclonal antibodies results in similar prevention of endothelial injury.³⁴ The improvement of graft function is thus mainly a result of its antineutrophil activity; vasodilating properties of NO play only a secondary role. These findings are in agreement with those of Naka and coworkers,³⁰ who could not reproduce the same lung protection achieved with the NO-donor nitroglycerin by adding hydralazine, a vasodilator, to the preservation solution. PMN accumulation was in-

hibited after nitroglycerin as well. A study of ischemic and reperfused myocardium by Lefer and colleagues²⁹ further supports our data. PMN accumulation was markedly diminished and blood flow was only modestly increased after administration of an NO donor at the time of reperfusion. Examination of the effects of short-term inhaled NO therapy on the transplanted lung after 24 hours of reperfusion enables us to support the hypothesis that NO does not merely delay the onset of pulmonary injury; rather, it seems to reduce the extent of cell injury.

Although the precise role of endogenous NO as a modulator of the acute inflammatory response is still a matter of debate, there is also evidence for a harmful interaction between NO and superoxide in IR injury.³⁵ NO and superoxide can combine to form peroxynitrite anion and hydroxyl anion, both of which have potent oxidizing capabilities.¹⁹ This potential raised some reservations about the use of inhaled NO in the setting of IR injury.³⁰ Although we did not measure any reactive oxygen metabolites in this experiment, we did not observe any adverse effects from toxic oxygen intermediates. In the study of Eppinger and associates³⁵ on pulmonary IR, adverse effects were seen early, after 30 minutes of reperfusion. After 4 hours, however, a protective effect was seen, and lung PMN content was significantly reduced, confirming our findings.

Previous *ex vivo* studies by our group showed a reduction in IR-induced endothelial dysfunction after supplementation with L-arginine or NO inhalation,^{17,18} and we tested this hypothesis *in vivo*. As a consequence of the significant lung injury created in our model, a deleterious effect on endothelium-dependent relaxation was seen in both groups. Only a tendency toward better relaxation was observed in the NO group. One limitation of this study is that grafts were stored for 24 hours in normal saline solution. There is convincing evidence that normal saline solution should not be used as a preservation solution in vascular operations because it seriously damages the endothelium.³⁶ The seemingly irreversible alteration of endothelial cell function observed after the long periods of IR set in our model could explain why the NO-induced reversibility of endothelial dysfunction seen in our previous studies and those of others^{17,18,37} was not found again. Relaxation responses from right native lungs were also altered to a much lesser degree than in the transplanted left lungs, however, confirming the notion that at reperfusion humoral factors damaging to the contralateral lung are released.^{3,31} In contrast with

Kimblad, Sjöberg, and Steen's results,¹⁴ endothelial dysfunction in our experiment did not correlate with PVR. Yet another limitation of our study is that data generated in segmental PAs rather than in microcirculatory vessels might not reflect the true vascular pulmonary impedance.

Our findings have direct clinical implications. In an era of widespread organ shortage, use of inhaled NO to attenuate IR injury and obtain a lasting beneficial effect may lead to strategies by which more marginal organs can be used. In conclusion, we found that 4 hours of inhaled NO therapy started at reperfusion significantly attenuates IR injury, improving graft function as long as 24 hours after operation. The blunting of IR injury is probably mediated by a decrease in PMN adherence and sequestration in both the transplanted and the contralateral lungs. These findings, combined with the lack of NO-related toxic side effects, make NO a suitable agent to use during the initial period of reperfusion after lung transplantation, specifically in cases where acute reperfusion phenomenon caused by poor graft quality or long ischemic periods is expected.

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