ALTERATIONS OF NITRIC OXIDE SYNTHASE EXPRESSION AND ACTIVITY DURING RAT LUNG TRANSPLANTATION

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Liu, Mingyao, Lorraine Tremblay, Stephen D. Cassivi, Xiao-Hui Bai, Eric Mourgeon, Andrew F. Pierre, Arthur S. Slutsky, Martin Post, and Shah Keshavjee. Alterations of nitric oxide synthase expression and activity during rat lung transplantation. Am J Physiol Lung Cell Mol Physiol 278: L1071–L1081, 2000.—Decreased nitric oxide (NO) production has been reported during lung transplantation in patients. To study the effects of ischemia and reperfusion on endogenous NO synthase (NOS) expression, both an ex vivo and an in vivo lung injury model for transplantation were used. Donor rat lungs were flushed with cold low-potassium dextran solution and subjected to either cold (4°C for 12 h) or warm (21°C for 4 h) ischemic preservation followed by reperfusion with an ex vivo model. A significant increase in inducible NOS and a decrease in endothelial NOS mRNA was found after reperfusion. These results were confirmed in a rat single-lung transplant model after warm preservation. Interestingly, protein contents of both inducible NOS and endothelial NOS increased in the transplanted lung after 2 h of reperfusion. However, the total activity of NOS in the transplanted lungs remained at very low levels. We conclude that ischemic lung preservation and reperfusion result in altered NOS gene and protein expression with inhibited NOS activity, which may contribute to the injury of lung transplants.

ischemia-reperfusion; acute lung injury; early graft dysfunction

ADVANCES IN SURGICAL TECHNIQUE and immunosuppression have transformed lung transplantation from an experimental procedure to a lifesaving therapy for patients with end-stage lung disease. However, the number of lung transplants that can be carried out remains severely limited by the shortage of suitable donor organs (10, 37). Furthermore, graft ischemic time must be limited to 4–6 h to minimize the incidence of early graft failure. Even with strict donor criteria and relatively short preservation times, severe ischemia-reperfusion injury is seen in ~20% of lung recipients and contributes significantly to the first-year postoperative mortality (13, 16).

Although the pathophysiology of ischemia-reperfusion injury is still not completely understood, loss of normal endothelial function and integrity appears to play a pivotal role (17). Numerous studies have demonstrated that lung preservation and reperfusion leads to an apparent deficit of nitric oxide (NO) (30), a key modulator of pulmonary vascular tone as well as of neutrophil and platelet adhesion (11, 21). Although exogenous NO may be given by inhalation to overcome the NO deficit, a concern exists because NO, especially in the presence of high oxygen concentrations, can react to form peroxynitrite, a strong oxidant that undoubtedly possesses toxicity and thus may increase graft injury (30). Despite the importance of both endogenous and exogenous NO in the context of lung transplantation, little is known about the expression and function of endogenous NO synthase (NOS) during the ischemia and reperfusion process. Better understanding of these changes will provide information at the molecular level regarding ischemia-reperfusion injury of the lung, and it could also provide clues as to how to properly use exogenous NO during lung transplantation.

Three different NOS isoforms are involved in the production of endogenous NO via the conversion of L-arginine to L-citrulline. Two of these isoforms are constitutively expressed, primarily in endothelial and neuronal tissue (eNOS and nNOS, respectively), and are responsible for many of the beneficial properties of NO such as reduced vascular tone, prevention of neutrophil and platelet adhesion, and neuronal transmission (11, 15). The third isoform, inducible NOS (iNOS), is induced at the transcriptional level by a number of stimuli including cytokines [such as tumor necrosis factor (TNF)-α]. iNOS has been implicated in the killing of exogenous organisms as well as in the pathophysiology of vascular collapse with septic shock, impaired hypoxic vasoconstriction, and tissue injury (11, 15).

In this study, we first used an ex vivo reperfusion model developed in our laboratory to examine the effects of injurious ischemic preservation conditions and subsequent reperfusion on NOS gene expression.
We then used an in vivo rat lung transplantation model, which more closely simulates the ischemia-reperfusion injury seen clinically without the confounding effects of a perfusion circuit, to examine gene expression, protein content, distribution of NOS isoforms, and total NOS activity. Our findings support a role for aberrant NOS expression and inhibition of NOS activity in the pathophysiology of lung injury related to preservation and reperfusion.

METHODS

Animal Care

Adult male Wistar rats and Lewis rats were used in this study. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the Institute of Laboratory Animal Resources (Toronto, ON), the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health [DHEW Publication No. (NIH) 86-23, Revised 1985], and the Guide to the Care and Use of Experimental Animals formulated by the Canadian Council of Animal Care (2nd ed., 1993).

Ex Vivo Lung Reperfusion Model

Donor operation and graft preservation. Adult male Wistar rats (350–400 g; Charles River, St. Constance, PQ) were used in the ex vivo reperfusion model. The lungs were harvested as previously described (9). Briefly, after intraperitoneal injection of pentobarbital sodium (40 mg/kg body wt; MTC Pharmaceuticals), the rats underwent tracheotomy and intubation with a 14-gauge endotracheal cannula and an intravenous injection of 500 U of heparin. They were ventilated with a Harvard ventilator (model 683, South Natick, MA) with room air at 70 breaths/min, tidal volume (VT) of 3 ml, and 3 cmH2O positive end-expiratory pressure (PEEP). A midline sternotomy was performed, the pulmonary arterial trunk was cannulated, and the left atrial appendage was transected followed by a pulmonary arterial flush with 100 ml/kg of a low-potassium dextran (LPD) solution (19) at a pressure of 15 cmH2O. The heart-lung blocks were removed, with the lungs inflated at end inspiration and stored for either 12 h at 4°C or 4 h at room temperature. These two preservation conditions were chosen based on previous studies (8, 9, 29) that revealed a similar and reproducible degree of lung injury after reperfusion. Lung tissue was snap-frozen immediately after the flush (mediastinal lobe) and after the preservation period (right lung).

Reperfusion. Reperfusion of the left lung was carried out with an established ex vivo model (9). Briefly, the lung being studied was placed in a circuit in which another fresh isolated lung was used as a deoxygenator by ventilating with a hypoxic gas mixture (4% O2-8% CO2-88% N2). The perfusion circuit was primed with 20 ml of fresh venous blood collected from heparinized rats. The entire perfusion circuit was housed in a warmed (36–38°C) and humidified Plexiglas chamber. The effluents from the deoxygenator and the study lung were continuously collected in two separate reservoirs and pumped to the other lung bloc via the main pulmonary artery (PA). Airway and pulmonary arterial pressures were continually monitored (Hewlett-Packard 8A multichannel recorder).

Blood gas analysis (CIBA Corning 278 blood gas system, Medfield, MA) was performed 10 min after reperfusion and then every 30 min. Before reperfusion was initiated, the preserved lung was first fully recruited by inflation to an airway pressure of 20 mmHg for 2 min followed by ventilation (40 breaths/min, 3 cmH2O PEEP, VT 1.5 ml, room air). The rate of perfusion was gradually increased from 0 to 10 ml·kg⁻¹·min⁻¹ over 10 min with a double-head roller pump (Cobe-Palmer, Chicago, IL) and then continued for 2 h, after which the left lung was snap-frozen for further analysis.

To assess whether reperfusion had a time-dependent effect on NOS gene expression and also to assess the contribution of reperfusion blood in the pulmonary vasculature to NOS mRNA levels, an additional group of cold-preserved lungs was studied. Six lungs were subjected to reperfusion for 0.5 h and others for 2 h. After reperfusion, three lungs at each time point were subjected to an intravascular flush with 100 ml/kg of a LPD solution at a pressure of 15 cmH2O before the tissue was frozen, while another three lungs were frozen immediately.

In Vivo Rat Single-Lung Transplant Model

To avoid allograft-related immune responses, male Lewis rats (300–350 g; Harlan, Indianapolis, IN) were used as both donor and recipient animals for isograft transplantation. Different from Wistar rats (an outbred strain), Lewis rats are from an inbred strain. Left single-lung transplants were performed with a cuff-anastomosis technique as described by Marck and Wilddevuur (23) with modifications (1, 26).

Donor operation and graft preservation. The donor operation procedure was similar to that described in Ex Vivo Lung Reperfusion Model, Donor operation and graft preservation. After flush preservation with 20 ml of a LPD solution and excision of the heart-lung bloc, the left hilar structures were isolated and dissected. A clamp (Sugita aneurysm clip, Mizuho, Burlington, ON) was placed on the bronchus to maintain inflation of the left lung during preservation. Samples were then taken from the right lung and snap-frozen in liquid nitrogen (“Flushed”). The left pulmonary artery and vein along with the left bronchus were transected proximally. These structures were then each attached to Teflon cuffs made from a 14-gauge angiocatheter (Becton Dickinson, Sandy, UT). The lung was then immersed in a LPD solution for a period of 4 h at room temperature. The remaining sections of right lung were similarly preserved and snap-frozen after a 4-h preservation (“Preserved”).

Recipient operation. Recipient rats were anesthetized with ketamine (Rogar, London, ON) and atropine (Ayerst, Montreal, PQ), intubated with a 16-gauge Teflon angiocatheter, and mechanically ventilated with room air at 75 breaths/min and a VT of 10 ml/kg. Anesthesia was maintained with 0.5–1.0% halothane. A midline sternotomy was performed, the right hilar structures were transected proximally. These anastomoses were then attached to the respective donor structures and their cuff. The native left lung was then released, followed by the pulmonary vein clamp and finally the pulmonary artery clamp. The native left hilum was then excised. The chest was closed with 5-0 prolene while the air in the left pleural space was evacuated via a 16-gauge butterfly catheter (Becton Dickinson).

Evaluation of transplanted lung. Two hours after the start of reperfusion, a laparotomy followed by a sternotomy was performed before the addition of 1 cmH2O PEEP and 100% O2 to the ventilator settings. The right hilum was clamped, and after a 10-min period for gas-exchange equilibration, an arterial blood gas sample was obtained from the abdominal aorta to evaluate the gas-exchange function of the transplanted lung. The right hilar clamps were then removed. A dose of 100 U of heparin was then injected into the inferior vena cava. After time was allowed for circulation of heparin,
the inferior vena cava was transected, the left atrial appendage was amputated, and the lungs were flushed via a catheter placed into the main PA with a 0.9% saline solution. The heart-lung bloc was excised after the saline flush, and samples from the left ("Transplanted") and right ("Native") lungs were snap-frozen for subsequent analyses (n = 5 transplants).

**RNA Extraction**

Total RNA was extracted from frozen tissue samples using the guanidinium isothiocyanate method described by Chomczynski and Sacchi (5). Briefly, frozen tissues (~0.1 g) were crushed in a liquid nitrogen-cooled mortar. The crushed powder was mixed with 5 ml of 4 M guanidinium thiocyanate buffer (GIBCO BRL, Life Technologies, Gaithersburg, MD) containing 1.5% of freshly added β-mercaptoethanol and homogenized with a Polytron tissue homogenizer (Brinkman, Mississauga, ON) for 1 min at medium speed. The lysate was mixed with 0.1 ml of 2 M sodium acetate, pH 4.2, followed by the addition of 5 ml of phenol, pH 4.5 (Amrasco, Solon, OH), and 1 ml of chloroform-isooamyl alcohol (49:1). The aqueous phase was collected after centrifugation at 8,000 rpm for 40 min at 4°C with a Beckman J-2-21 high-speed centrifuge (J A-17 rotor, Beckman, Fullerton, CA). The supernatant was transferred to a new tube, and RNA was precipitated by mixing with an equal volume of isopropanol. RNA concentration was measured with a Beckman DU 640B spectrophotometer.

**Semiquantitative RT-PCR**

In our pilot studies, we noted that heparin, used as an anticoagulant during the surgical operation, interfered with RT-PCR. The inhibitory effect can be overcome with a heparinase treatment (data not shown). RNA samples (4 µg) were reverse transcribed using a SUPERSCRIPT II kit (GIBCO BRL) according to the manufacturer's instructions. Reverse transcription of total RNA from each sample was done with a SUPERSCRIPT II kit (GIBCO BRL) according to the manufacturer's instructions. Further amplification of specific genes by PCR was carried out with 10% of the RT product each time.

Using Northern blotting and semiquantitative RT-PCR, we found that the steady-state mRNA levels of β-actin were not affected by lung preservation and transplantation (data not shown). The message level of β-actin was therefore used as an internal control to assess the quality and quantity of RNA extraction and efficiency of the RT-PCR. The primers for β-actin, NOS isoforms, and TNF-α were synthesized based on published sequences (see Table 1). The total volume of each PCR mixture was 30 µl containing 3 µl of 10× PCR buffer (200 mM Tris-Cl, pH 8.4, and 500 mM KCl), 1 µl of 50 mM MgCl2, 0.7 µl of 10 mM deoxynucleotide triphosphate mix, 0.5 µl of each PCR primer (10 µM), and 0.3 µl of Taq polymerase (GIBCO BRL). PCR was performed with a programmable thermal cycler (PCT-100, MJ Research, Watertown, MA). The PCR conditions were optimized for each molecule and are summarized in Table 1.

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<th>No. of PCR Cycles</th>
<th>Annealing Temperature, °C</th>
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Quantitative RT-PCR

To determine the accuracy of semiquantitative RT-PCR, steady-state mRNA levels of TNF-α were also analyzed with an ELISA-based quantitative PCR detection kit (Biosource, Camarillo, CA). A known copy number of an exogenously synthesized DNA containing PCR primer binding sites identical to TNF-α CDNA, an internal calibration standard (ICS), was mixed with the sample cDNA before amplification. One of the TNF-α primers was biotinylated and incorporated into both the ICS and TNF-α amplicons. After PCR, the amplicons were denatured and hybridized to either ICS or TNF-α sequence-specific capture oligonucleotides prebound to microwells for ELISA. The ICS was amplified at an efficiency identical to the TNF-α cDNA and thus served as a standard for TNF-α cDNA quantification. To find the appropriate ratio between TNF-α and ICS, cDNA from each sample underwent a series of dilutions. The PCR contains 10 µl of 10× PCR buffer, 3 µl of 50 mM MgCl2, 2 µl of 10 mM deoxynucleotide triphosphates, 2 µl of TNF-α primers, 5 µl of 400 copies/µl of ICS, 5 µl of diluted cDNA, 0.5 µl of Taq polymerase, and 73 µl of H2O. The optimized PCR condition was 95°C for 5 min, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C for 7 min for 30 cycles. Ten microliters of PCR product were analyzed by 1% agarose gel electrophoresis. Only the PCRs that showed both TNF-α and ICS amplicons were chosen for ELISA detection following the manufacturer's instructions. The final results were calculated and are...
expressed as the copy number of TNF-α cDNA per microgram of total RNA.

Immunohistochemistry Staining for NOS Isoforms

Two hours after single-lung transplantation, the lungs were flushed (via a catheter placed into the main PA) with 4% paraformaldehyde in phosphate-buffered saline (PBS) for fixation under a constant airway pressure of 12 cmH2O (n = 3 transplants). The Transplant (left) and Native (right) lung lobes were embedded in paraffin and cut into 5-μm sections. The sections were mounted on α-aminopropyltriethoxysilane-coated slides. An avidin-biotin immunoperoxidase method was used as previously described (12) to stain the NOS isoforms. Endogenous peroxidase enzyme activity was quenched with 1% hydrogen peroxide in methanol. Non-specific binding sites were blocked with 5% (vol/vol) normal goat serum and 1% (wt/vol) bovine serum albumin (BSA) in PBS. Excess blocking solution was removed, and the tissue sections were incubated overnight at 4°C with rabbit anti-NOS antibodies (Affinity Bioreagents, Golden, CO). Tissue sections were washed with PBS three times and incubated with biotinylated secondary goat anti-rabbit IgG (1:600 dilution). After being washed with PBS, the sections were incubated with an avidin-biotin complex for 2 h. The sections were washed again with PBS and developed in 0.075% (wt/vol) 3,3′-diaminobenzidine in Tris-HCl buffer, pH 7.6, containing 0.002% (vol/vol) hydrogen peroxide. The slides were slightly counterstained with Carazzi’s hematoxylin, dehydrated in an ascending ethanol series and then in xylene, and mounted. In control staining, specific primary antibodies were replaced with nonimmune rabbit IgG or with blocking solution only.

Western Blotting Analysis

To determine protein contents of NOS isoforms, the frozen tissues were homogenized with radioimmunoprecipitation assay buffer. The tissue lysates were sonicated for 5 s twice on ice and centrifuged at 12,000 g for 5 min at 4°C, and the supernatant was collected and stored at −70°C before analysis. Protein concentration was determined by a standard protein assay (Bio-Rad Laboratories, Richmond, CA). Equal amounts of total protein (150 μg) were boiled with SDS sample buffer [10% (vol/vol) glycerol, 2% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, 0.0025% (wt/vol) bromphenol blue, and 0.06 M Tris, pH 8.0] and subjected to 6% SDS-PAGE. The proteins were transferred to nitrocellulose membranes. Non-specific binding was blocked by incubation of the membranes with 10% (wt/vol) BSA in PBS overnight at room temperature. The blots were incubated with a rabbit polyclonal anti-iNOS or anti-nNOS antibody or a monoclonal anti-eNOS antibody (Transduction Laboratories, Lexington, KY) and diluted at 1:300, 1:500, or 1:500, respectively, in PBS containing 5% BSA and 10% goat serum. The blots were incubated at room temperature for 90 min, washed with PBS, and incubated for 90 min at room temperature again with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:30,000 dilution) or goat anti-mouse IgG (1:2,500 dilution) in 1% BSA and 10% goat serum. After being washed, the blots were developed with an enhanced chemiluminescence detection kit (Amersham, Oakville, ON).

NOS Activity Assay

Total NOS activity was measured with a NOSdetect assay kit from Stratagene (La Jolla, CA) following the manufacturer’s instructions. Briefly, the frozen lung tissues were homogenized in 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 1 mM EGTA. Protein concentration was measured as described in Western Blotting Analysis. Tissue lysate containing 200 µg of protein was combined with a reaction mixture containing 25 mM Tris-HCl, pH 7.4, 3 mM tetrahydrobiopterin, 1 mM FAD, 1 mM flavine adenine mononucleotide, 1 mM NADPH, 0.6 mM CaCl2, 0.05 µCi of L-[14C]arginine (Amersham, Arlington Heights, IL) and incubated at room temperature for 30 min. After the reactions were stopped with stop buffer, equilibrated resin was added to each reaction sample, transferred to spin cups, and centrifuged at full speed for 30 s with a microcentrifuge. The radioactivity of eluate (containing citrulline) was quantified with a liquid scintillation counter. The NOS activity was calculated and is expressed as picomoles per minute per milligram of protein or as percentage of citrulline converted.

Statistical Analysis

Results are presented as means ± SE. Statistical analysis was carried out with SigmaStat for Windows, version 1.0 (Jandel, San Rafael, CA). Comparison between two groups was assessed by Student’s t-test. Comparisons of more than two groups were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls correction for multiple range comparison. P values < 0.05 were considered significant.

RESULTS

Ischemia-Reperfusion Induced Changes in iNOS and eNOS mRNA Levels in an Ex Vivo Reperfusion Model

An ex vivo reperfusion model was developed in our laboratory to evaluate the function of rat lung grafts after various preservation conditions (9). This model permits reliable assessment of pulmonary function in rats under controlled ventilation and perfusion conditions. Using this model, DeCampos and colleagues have previously shown that rapid reperfusion induced a reproducible injury of ischemic rat lungs preserved at room temperature for 4 h (9) or at 4°C for 12 h (8), with decreased arterial P02 (P02s) and increased shunt, peak airway pressure, and pulmonary edema. Similar pathological changes were observed in the present study (data not shown). Thus these preservation conditions were used to test the effects of ischemia-reperfusion injury on NOS gene expression.

Compared with the normal control lung, the steady-state mRNA levels of iNOS remained the same after flushing and preservation. Two hours after reperfusion, iNOS mRNA levels increased significantly in both cold- and warm-preserved lungs (Fig. 1). In contrast, a decrease in eNOS mRNA was found after 2 h of reperfusion after warm preservation relative to either freshly harvested (control) or Flushed lungs (Fig. 2A). A relatively lower level of eNOS mRNA was also noted in lungs subjected to cold preservation followed by 2 h of reperfusion compared with that in Flushed lungs (Fig. 2A). No change in nNOS mRNA was observed after flush, preservation, or reperfusion (Fig. 2B).

To determine whether the changes in iNOS and eNOS mRNAs are related to the intravascular blood elements reintroduced into the lung during reperfusion, lung tissues were collected after either 0.5 or 2 h of reperfusion. At each time point, a subgroup of lungs was flushed to remove blood from the pulmonary
vasculature. As depicted in Fig. 3, the induction of iNOS was found to increase with the duration of reperfusion and was not affected by removal of intravascular blood before tissue assessment. In this experiment, no significant change in eNOS mRNA was observed (Fig. 3).

Ischemia-Reperfusion Induced Changes in iNOS and eNOS mRNA Levels in an In Vivo Lung Transplant Model

To determine whether the observed changes in NOS gene expression were related to ischemia-reperfusion or to the perfusion of isolated lungs in a circuit, we repeated the experiments in a rat single-lung transplantation model. To avoid acute allograft rejection-related immune responses, isograft lung transplantation was performed between inbred Lewis rats. Because warm and cold preservation conditions employed in the first stage of the studies demonstrated a similar degree of acute injury of the lung (8, 9) and similar changes in NOS gene expression, only warm preservation was used in this series of experiments. To evaluate the function of the transplanted left lung, blood gas samples were taken after the hilum of the recipient’s native lung was clamped for 10 min. The PaO₂ of the isograft recipients ventilated with 100% O₂ fell from 577 ± 38 mmHg before transplantation to 254 ± 49 mmHg after 2 h of reperfusion. Interstitial pulmonary edema was observed with light microscopy (see Ischemia-Reperfusion Increased iNOS and eNOS Protein Contents in Transplanted Lungs). The drop in PaO₂ and the pathological changes indicate a moderate degree of lung injury because when the rat lungs preserved at 4°C for 6 h were transplanted, normal PaO₂ and lung histology were noted (data not shown).

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Similar to the ex vivo model, a significant increase in iNOS mRNA was found in the transplanted lung 2 h after reperfusion (Fig. 4). Of note, no changes were found in the recipients’ native lungs, supporting the notion that the increase in iNOS observed in the transplanted lung is a result of a specific ischemia-reperfusion-related response rather than a generalized systemic inflammatory response. After reperfusion, a decrease in eNOS mRNA was observed, but it was not significant. Once again, there was no change in nNOS mRNA in any of these experimental settings (Fig. 4).

Ischemia-Reperfusion Increased iNOS and eNOS Protein Contents in Transplanted Lungs

The localization and expression of NOS proteins in the parenchyma of Transplant and Native lung tissues were examined with immunohistochemical techniques. Varying degrees of interstitial edema were noted in the Transplant lungs (n = 3), and no significant alveolar macrophage or neutrophil infiltration or hemorrhage was found in the alveolar spaces. The Native lung had a relatively normal appearance as examined with light microscopy. The immunohistochemical staining of iNOS was clearly increased in the Transplant lungs compared with that in the paired Native lungs from the same experiment (Fig. 5, A and B). Positively stained cells were localized on the septa of the alveolar wall, especially at the corners of the alveolar spaces. The distribution of positive staining is consistent with the distribution of alveolar type II epithelial cells (Fig. 5, A and B). Interestingly, we found that staining of eNOS was also significantly increased in the Transplant lung, although clear staining in the Native lung was also observed. The location of positively stained cells was along the alveolar wall, consistent with the distribution of the endothelial cells (Fig. 5, C and D). Weak staining for nNOS was detectable in the lung parenchyma; however, no difference was seen between Transplant and Native lungs (Fig. 5, E and F). When the first antibody was replaced with rabbit nonimmune IgG or omitted from the staining protocol, no staining was observed (data not shown). These results were consistent in all three separate experiments.

We then examined the content of NOS proteins in the in vivo lung transplant model with Western blotting analysis. The iNOS protein was almost undetectable in the Flushed and Preserved lung tissues (data not shown). It was significantly increased after 2 h of reperfusion (Fig. 6A). Compared with that in the Transplant lung, the iNOS level in the Native lungs of recipients was almost undetectable (Fig. 6A). The total amount of eNOS protein in the Transplant lungs was significantly higher than that in the Native lungs (Fig. 6, B and C). No measurable amount of nNOS was found with the immunoblotting conditions used (data not shown), suggesting that in comparison with eNOS, the level of nNOS in the lung is very low.

NOS Activity Was Not Altered by Ischemia and Reperfusion

We then examined the total NOS activity from Flushed, Preserved, Transplant, and Native lungs. For comparison, lung tissues from normal rats and rats injected with lipopolysaccharide as positive controls (36) were included. The NOS enzymatic activity was evaluated by the conversion of L-[14C]arginine to L-[14C]citrulline. The conversion rate in normal rat lungs was ~1.5% and in lipopolysaccharide-challenged rat lungs was ~10%. These were both similar to that reported by other investigators (20). The NOS activity was ~0.25 pmol·min⁻¹·mg protein⁻¹ in normal rat lungs, which is also in the same range as that reported for cultured human lung epithelial cells (3). Interestingly, although both iNOS and eNOS protein levels were increased in Transplant lungs, total NOS activity remained at a very low level as that in the other groups (Table 2).

Ischemia-Reperfusion Induced TNF-α Gene Expression During Lung Transplantation

It is known that the expression of iNOS can be upregulated by proinflammatory cytokines such as TNF-α in the lung. TNF-α is known to increase after
ischemia and reperfusion (32, 33). To determine whether the increase in iNOS is associated with a change in TNF-α, the steady-state mRNA levels of TNF-α were measured by semiquantitative RT-PCR. A significant increase in TNF-α mRNA was found at the end of the warm preservation period and after reperfusion (Fig. 7A). After transplantation, a significant increase in TNF-α mRNA in the Native lung of the recipients was also observed, although the level was significantly lower than that in the Transplant lung (Fig. 7A).

Using a recently available quantitative RT-PCR kit for TNF-α, we quantified the copy number of TNF-α mRNA transcripts from lung tissues after preservation and transplantation. The observed increase was on the same order (Fig. 7B) as we found with the semiquantitative RT-PCR method (Fig. 7A). For example, the increment in TNF-α mRNA was ~6-, 8-, and 3-fold as measured by semiquantitative PCR and was ~8-, 9-, and 3-fold as determined by quantitative PCR from Preserved, Transplant, and Native lung tissues, respectively. This comparison suggests that using semiquantitative RT-PCR to evaluate the changes in steady-state levels of mRNA can provide reasonable information, especially when the changes are as dramatic as those seen with iNOS in the present study.

**DISCUSSION**

In this study, we examined expression of the NOS isoforms responsible for the production of NO with lung injury models of transplantation-related preservation and reperfusion. We found a significant increase in iNOS mRNA and increased iNOS and eNOS proteins after reperfusion in the transplanted lung, but total NOS activity remained unchanged. These results suggest alterations in NOS gene expression, protein synthesis, and inhibition of NOS activity associated with ischemia and reperfusion injury of lung transplants.

**NO, NOS, and Lung Transplantation**

NO, an important molecular mediator, is produced by a variety of cell types and is involved in numerous physiological processes in the lung including pulmonary vasoregulation, smooth muscle relaxation, inhibition of platelet aggregation and neutrophil adhesion, and maintenance of integrity of the endothelium (11, 15). Decreased endogenous NO production in the transplanted lung has been found in rats (30) and in patients (24) during lung transplantation. However, the mechanisms of reduced NO production are unknown. The transplanted lung retains its ability to respond to exogenously administrated NO. In fact, inhalation of
NO as a therapeutic modality has been successfully employed in patients after bilateral lung transplantation in our Lung Transplantation Program and in other programs (6) to improve oxygenation and to reduce human lung allograft dysfunction. As discussed in a recent review (14), a major potential limitation of NO application during lung transplantation is the interaction between the exogenous NO and the endogenous NOS systems. The potential toxicity related to the interaction of NO with reactive oxygen species at the time of reperfusion may also be problematic. Furthermore, although transplanted lung function after exogenous NO administration can, in some instances, be improved dramatically in our experience and in that of others (6), it remains impossible at this time to predict which patients will respond to NO treatment.

Alterations of NOS expression have been reported at various stages after lung transplantation. For example, several days after transplantation, increased iNOS expression was demonstrated in an experimental acute lung allograft rejection model (40), and inhibitors of iNOS have been shown to ameliorate rat lung allograft rejection (34, 39). Increased iNOS and decreased eNOS expression were found by immunohistochemical staining in airway epithelium and vascular endothelium from patients with obliterative bronchiolitis (25), the most important chronic complication after lung transplantation. However, the regulation of NOS gene expression, protein synthesis, and activity has not been previously reported during lung preservation and reperfusion.

**Ischemia-Reperfusion Induced Alteration of NOS Expression**

Despite strict donor criteria and restriction of cold ischemic times to <4–6 h, ischemia-reperfusion injury remains a major cause of early morbidity and mortality in lung transplant recipients (37). The hallmark findings of ischemia-reperfusion injury include progressive deterioration in gas exchange, opacification of the chest X ray, neutrophil infiltration, and increased pulmonary vascular resistance (37). Although the precise mechanisms remain unclear, impaired endothelial and lung epithelial functions as a result of lung ischemia and reperfusion are thought to be involved, and the impairment of the NOS system may play an important role.
(14). The fact that some lung transplant patients can have severe hypoxia that is more than expected given the degree of pulmonary edema seen on their chest X ray led us to suspect that an aberrance of the NOS system leading to impaired vasoregulation may be a fundamental underlying injury in the transplanted lung.

Decreased endogenous NO production from isograft lung transplants has been demonstrated (30), suggesting that the expression and activity of NOS may be altered by ischemia-reperfusion. In the present study, we found a dramatic increase in iNOS mRNA expression in both the ex vivo and the in vivo models after lung reperfusion. The increase in iNOS mRNA was time dependent, not affected by flushing the blood from the pulmonary vasculature at the end of reperfusion, and restricted to the transplanted isograft in vivo. Immunohistochemistry and immunoblotting studies confirmed that the increased iNOS mRNA was accompanied by increased iNOS protein expression by pulmonary parenchymal cells after reperfusion. The absence of changes in the contralateral native lung of the recipients argues against a nonspecific increase in iNOS secondary to a systemic inflammatory response. Because isograft transplantation was performed in this study, the upregulation of iNOS must be a consequence of ischemia-reperfusion rather than an immunemediated response such as that seen in the acute allograft lung rejection situation (40).

In both ex vivo and in vivo models, mRNA levels of eNOS were either slightly decreased or unchanged after ischemia-reperfusion. Several studies indicate that constitutive NOS expression in endothelial cells can be elevated by fluid shear stress (31, 38) or cyclic mechanical strain (4). The process of lung transplantation presents a unique situation in which both shear stress and mechanical strain are reintroduced to the lung tissues after preservation. Dramatic changes in these physical forces play an important role in reperfusion-induced lung injury during transplantation (7–9, 29). The lack of an increase in eNOS mRNA may represent the relative inhibition of the normal endothelial cell response to these physical stimuli. Because an increase in iNOS mRNA was found, global impairment of gene transcription after reperfusion is an unlikely explanation. We speculate that ischemia and reperfusion may selectively suppress the endothelium to appropriately respond to shear stress. Although eNOS mRNA did not change very much, eNOS protein was elevated after reperfusion in the transplanted lungs. This result was unexpected. In general, it is thought that constitutive NOS activity is regulated at the protein level by calcium/calmodulin-dependent mechanisms (15). Based on our results, we speculate that that during lung transplantation, ischemia-reperfusion may have a direct regulatory effect on eNOS protein synthesis and/or degradation.

An apparent deficit of NO has been implicated in the pathophysiology of early lung graft injury after ischemia and reperfusion (30). Pinsky et al. (30) demonstrated that NO levels measured at the pulmonary surface plummeted immediately on reperfusion of rat lung isografts. Because no difference in lung homograft NOS activity was found, these investigators concluded that rapid consumption of NO, possibly by reactive oxygen intermediates, led to the reduced levels of NO. In the present study, we also found that total NOS activity did not change but that both iNOS and eNOS proteins were increased, suggesting the existence of endogenous NOS inhibitors in the lung that may be produced during transplantation. This mechanism may also account for the apparent deficit of NO observed clinically after lung transplantation. The production of NO was not measured directly in this study. However, using the same lung-to-lung perfusion model, Kawashima et al. (18) have shown that addition of an NO donor to lung preservation prevented reperfusion-induced lung injury. Therefore, the lack of endogenous NO production may play an important role in the graft injury seen in this model. It is also possible that the increased NOS proteins contain a large fraction of premature or inactive forms or lack cofactors for NOS activation.

The lack of change in NOS activity may be specific to lung transplantation-related ischemia-reperfusion because total NOS activity has been shown to rapidly increase after 2 h of ischemia-reperfusion in a rat renal model (35). In an isolated blood-perfused rat lung model, which simulated clinical situations such as reperfusion after removal of a massive pulmonary embolus or cardiopulmonary bypass, ischemia (30 min) and reperfusion (180 min) upregulated iNOS mRNA and activity but downregulated constitutive NOS activity (22). However, these experimental conditions are different from the hypothermic and aerobic preservation in lung transplantation.

How iNOS gene expression and protein synthesis are regulated during ischemia-reperfusion is unknown. We found an increase in TNF-α mRNA after warm ischemic preservation and after reperfusion from the transplanted lung. TNF-α is one of the cytokines known to induce iNOS expression (11, 15, 21). We found increased TNF-α gene expression after warm ischemia as determined by both semiquantitative and quantitative RT-PCR. The stress of lung harvest and warm ischemia may induce TNF-α expression. However, the increased TNF-α mRNA during warm preservation was not associated with the change in iNOS mRNA. In addition, a fair amount TNF-α transcripts were observed in the Native lung of the recipients, which was also not in parallel with the change in iNOS expression. Therefore, the change in TNF-α expression alone does not account for the change in iNOS gene expression seen after reperfusion of the transplanted lung.

Critique of Models Used in the Present Study

Both an ex vivo and in vivo rat model of lung preservation and reperfusion injury were used to elucidate the events responsible for changes in NOS expression. In the ex vivo model, a number of confounding factors inherent to in vivo models were absent, including the influx of systemically derived mediators and...
hemodynamic instability. The ex vivo model permitted assessment of the changes in eNOS expression under conditions of carefully controlled reperfusion. Limitations of this model, however, include the activation of circulating leukocytes and complement secondary to the tubing in the circuit, although ~30% of patients are placed on cardiopulmonary bypass during lung transplantation, and, therefore, this model is potentially very relevant.

An in vivo model of single-lung transplantation was also used to avoid the confounding factors mentioned above. These experiments confirmed that our findings were indeed related to the transplant ischemia-reperfusion process and not an alteration due to perfusion through an artificial circuit. To study the effect of ischemia-reperfusion on NOS expression and activity, isograft transplantation was performed to simplify the experimental condition. However, the impact of an allogenic immune response during the ischemia-reperfusion period should also be considered. A limitation of both models relates to species differences in susceptibility to ischemia-reperfusion injury, inflammatory responses, and gene regulation. As such, further studies in humans will ultimately be needed.

The gene expression of NOS isoforms was analyzed with semiquantitative RT-PCR. Ideally, the number of gene transcripts should be quantified. However, this approach is limited by the availability of quantitative PCR kits. We compared these two methods with TNF-α expression and found a very good correlation. Thus although the less dramatic changes in eNOS need to be interpreted cautiously, the dramatic increases in iNOS and TNF-α are convincing.

In summary, the transplanted lung appears to have a decreased bioavailability of NO. We have shown that iNOS gene expression is increased after reperfusion and that iNOS and eNOS protein expression are also increased but that total NOS activity remained unchanged. Clearly, these alterations are complex, and further studies are required to determine the detailed nature of these changes. By knowing how NOS expression and activity is regulated, we will be able to more precisely manipulate the NO system in the lung to the ultimate benefit of our lung transplant patients.

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