MODULATION OF REPERFUSION INJURY AFTER SINGLE LUNG TRANSPLANTATION BY PENTOXIFYLLINE, INOSITOL POLYANIONS, AND SIN-1

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Objective: Previous studies have suggested reductions in lung reperfusion injury with pentoxifylline, inositol polyanions, and the nitric oxide donor, SIN-1, but these agents have never been directly compared to ascertain which is superior. We investigated these agents in a porcine model of left single lung transplantation. **Methods:** Donor lungs were preserved with modified Euro-Collins solution for a mean ischemic time of 18.4 hours. Neutrophil trapping in the graft, pulmonary vascular resistance, free radical release (measured by malonaldehyde levels) and gas exchange were assessed over a 12-hour period. All groups were reperfused at an initial pulmonary artery pressure of 20 mm Hg. Group A (n = 5) was a control group with no interventions added; group B was reperfused with the addition of intravenous inositol polyanions (0.02 mg/kg/h), and group C was reperfused with intravenous SIN-1 (0.02 mg/kg/h). Group D was reperfused with the addition of intravenous pentoxifylline (2 mg/kg/h). **Results:** Neutrophil sequestration was observed within 10 minutes of reperfusion in group A. This was attenuated significantly by interventions in groups B, C, and D. In group D, malonaldehyde levels were significantly lower than in other groups and was associated with superior oxygenation. Pulmonary vascular resistance was reduced in groups B, C, and D compared with group A. **Conclusions:** Pentoxifylline, when administered only to recipient animals was superior to the other interventions studied. Inositol polyanions are promising as a possible therapeutic intervention but were not as effective as the other agents studied. (J Thorac Cardiovasc Surg 1999;117:556-64)

Despite advances in lung preservation technology, early graft dysfunction remains a significant problem. Lung ischemia-reperfusion injury is complex and multifactorial but principally involves the sequestration of activated neutrophils within the lung on reperfusion with subsequent release of injurious granular enzymes and oxygen free radicals.¹

Several interventions have been suggested to modu-

late reperfusion injury but have never before been directly compared in a single standardized model. Some of the more promising interventions include pentoxifylline, inositol polyanions (typified by inositol hexakisphosphate [InSP₆]), and the potent nitric oxide donor, SIN-1 (3-morpholiniosydnonimine).

Pentoxifylline administration has been successful in ameliorating reperfusion injury after skeletal muscle ischemia and experimental lung and liver transplantation. It acts through a variety of mechanisms including inhibition of leukocyte-endothelial interactions and oxygen free radical scavenging.²⁻⁵

Nitric oxide is a well-known autocrine and paracrine cellular mediator and prime moderator of pulmonary vascular physiologic condition. It is thought to be beneficial when administered by inhalation or intravenous routes (through nitric oxide-donating agents) to either donor or recipient. In addition to its involvement in vascular homeostasis, nitric oxide is also a naturally occurring inhibitor of neutrophil to endothelial adhesion.^{6,7}

InSP₆ is a phytic acid derivative that acts as a scav-

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enger of oxygen free radicals. There is much evidence from rodent models that this agent may also influence neutrophil-endothelial interactions by inhibiting L and P selectin-mediated adhesion.⁸

We investigated the actions of these agents and were uniquely able to directly compare their effects in a porcine model of single lung transplantation with follow-up for 12 hours. This enabled us to assess which intervention might be superior for subsequent use in clinical practice.

Methods

Twenty female Landrace pigs (mean weight, 45.4 kg) were divided randomly into 4 groups (n = 5 in each group). A similar number were size and weight matched and acted as donor animals. All grafts were reperfused at a mean pulmonary artery pressure of 20 mm Hg.

Group A was a control group with no intervention administered; group B was reperfused with intravenous inositol hexakisphosphate (0.02mg/kg/h), and group C was reperfused with intravenous SIN-1 (0.02mg/kg/h). Group D was reperfused with the addition of intravenous pentoxifylline (20 mg/kg loading dose, then 2mg/kg/h). All interventions were administered to the recipient animal only and began 5 minutes before reperfusion of the graft.

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 National Academy of Science and published by the National Institutes of Health (NIH publication 85-23, revised 1985). All conditions associated with the United Kingdom Animals (Scientific Procedures) Act 1986 were also met.

Donor operation. Animals were anesthetized with initial intramuscular premedication with diazemuls (2 mg/kg) and ketamine (15 mg/kg). Subsequent anesthesia was induced with propofol (20 mg/kg) and maintained with isoflurane and intravenous alfentanil. Animals were intubated with an endotracheal tube (outside diameter, 9.5 mm) and ventilated at a tidal volume of 15 mL/kg at an inspired oxygen concentration of 100%.

Heart-lung blocks were retrieved in a standard manner,⁹ and lungs were preserved by flush perfusion with 60 mL/kg of modified Euro-Collins through the main pulmonary artery.

After the separation of the left lung from the block, a pulmonary artery pressure monitoring line (Cavafix Certo 18G; Braun, Melsungen, Germany) was inserted through a purse-string suture into the distal left pulmonary artery. A pulmonary venous sampling line (Flocare; Nutricia, Madrid, Spain) was similarly placed through the left atrial cuff directed into a distal pulmonary vein, allowing for later sampling of venous blood from the graft without mixing from the contralateral native lung after transplantation and reperfusion. Lungs were stored inflated at a temperature of 4°C for a mean ischemic time of 18.4 hours.

Recipient operation. Recipient animals were premedicated with intramuscular azaperone (8 mg/kg) and diazemuls (2 mg/kg). After induction of anesthesia with intravenous propofol, animals were maintained on intravenous pentobarbitone (30 mg/kg/h) and alfentanil. Venous and arterial pressure monitoring lines were inserted as in donor animals.

Two endotracheal tubes were inserted through a tracheostomy. A 9.5-mm outside diameter tube was placed into the trachea to ventilate both lungs initially and subsequently the native lung alone. The second 6.5-mm tube was advanced through the left bronchial anastomosis after its completion to ventilate the graft lung independently. Each endotracheal tube was connected to a separate ventilator to permit individual lung ventilation after the transplantation, with a tidal volume of 15 mL/kg at 12 breaths per minute for each lung.

A left thoracotomy was followed by left pneumonectomy. Implantation of the donor lung proceeded in an established fashion constructing anastomoses of the left atrium, bronchus, and left pulmonary artery in order. The contralateral pulmonary artery was encircled by a tape and snigger such that the left pulmonary artery pressure could be manipulated. A pressure monitoring line was placed in the recipient left atrium, and a dedicated sampling line was inserted into the proximal left pulmonary artery.

Pulmonary artery flow was measured with a 10-mm flow probe (Transonic A-Series; Linton Instruments, Norfolk, UK) placed around the left pulmonary artery distal to the anastomotic line. A similar 12-mm probe was placed around the descending aorta to provide a guide to the cardiac output. Both flow probes were connected to a dual channel HT207 medical volume flowmeter (Transonic Systems Inc, Ithaca, NY).

All of the pressure and flow data sources were routed through a CED 1401 32 channel digital to analogue converter (Cambridge Electronic Design Ltd, Cambs, UK) and acquired on a Gateway 2000 PC (Gateway 2000 Europe, Clonslough, Dublin, Ireland) running Microsoft Windows 95 (Microsoft Ltd, Workingham, Berks, United Kingdom) and Spike 2 (Version 4.0) data acquisition software (Cambridge Electronic Design Ltd, Cambs, United Kingdom). Data were collected continually over the 12-hour period after the operation and stored on a hard disk for subsequent analysis.

Pulmonary venous oxygen partial pressure (millimeters of Mercury) was obtained from pulmonary venous sampling line samples analyzed immediately on a blood gas analyzer (Stat Profile 5, Nova Biomedical, Waltham, Mass).

Pulmonary vascular resistance (mm Hg/L/min) was calculated from the formula: (mean pulmonary artery pressure [mm Hg]) – (left atrial pressure [mm Hg])/left pulmonary artery flow (L/min).

Neutrophil trapping within the lung was calculated by expressing the neutrophil count of blood exiting the lung, measured from the sampling catheter in the graft pulmonary vein, as a percentage of that entering the lung (measured from the pulmonary artery sampling line) at each assessment interval. Neutrophil sequestration was determined as a percentage of the neutrophil count in pulmonary venous blood compared

Fig 1. Neutrophil sequestration after administration of pentoxifylline, SIN-1, and InSP₆. Results are shown as the mean value for animals in each intervention group (n = 5; group A, control; group B, inositol polyanions; group C, SIN-1; group D, pentoxifylline). Error bars indicate the 95% confidence interval. Pre-reperfusion value is shown at time 0. Note the significant attenuation of neutrophil sequestration in the intervention groups compared with controls. Mean absolute value at time 0 = $6.4 \times 10^9/L$.

with the pulmonary arterial blood at a particular time point. Thus values less than 100% indicate neutrophil trapping in the lung, although values exceeding 100% indicate neutrophil release from the lung.

Malonaldehyde, an important decomposition product of lipid peroxides, is an indirect measure of free radical activity.

A spectrophotometric assay with an LPO-586 method (Calbiochem-Novabiochem International, San Diego, Calif) was used to quantify malonaldehyde in pulmonary venous blood. Condensation of one molecule of malonaldehyde with 2 molecules of a proprietary chromogenic agent produces a stable chromophore with maximal absorbance at 586 nm.

Five-milliliter samples of whole blood were collected in 48 μL of potassium EDTA 0.17 mmol/L. After centrifugation at 2500g for 10 minutes at 4°C, 200 μL of the supernatant was collected in duplicate for use in the assay.

The sample was incubated for 40 minutes at 45°C with the chromogenic substance then cooled on ice, and absorbance was measured by spectrophotometer at 586 nm. The sensitivity of this method was determined as 0.5 $\mu mol/L$. In whole blood samples of 200 μL , the lower limit of measurable malonaldehyde was 2.5 $\mu mol/L$. Reproducibility was determined by assays done over 10 days under identical experimental conditions. With standard concentrations from 0 to 20 $\mu mol/L$, standard deviation was less than 5%.

Statistical methods. Although widely practiced, data analyzed using a 1-way analysis of variance to compare group means at a series of time points (with the null hypothesis that

there is no difference between the group means at each point) is flawed. Graphically, a line joining the mean values together may not describe individual subjects within a study group adequately so that a misleading impression is gained of the trends in the data. No account is taken of the fact that measurements at different time points are from the same subjects with successive observations of an individual subject likely to be correlated.¹⁰ To overcome these potential problems, statistical examination using the individual, rather than the mean of the group at each time point, as the basic unit for analysis can be performed. A single number to summarize an individual subject's response curve with time must be sought. This reduces a large number of dependent observations to a smaller number of summary measures.¹¹ Time-related trends in variables measured in reperfusion injury of the lung may be either peaked or of growth type where the trend is increasing or decreasing with time, such as the pulmonary venous oxygen tension.

In our study, the summary measure of area under the curve was used to describe the behavior of individual animals. Groups of animals were then compared with the Scheffé analysis of variance.

Results

Sequestration in the lung graft was observed in the first 10 minutes after reperfusion in control group A, with maximal sequestration occurring at 2 minutes.

Fig 2. Platelet sequestration after administration of pentoxifylline, SIN-1, and InSP₆. Results are shown as the mean value for animals in each intervention group (n = 5; group A, control; group B, inositol polyanions; group C, SIN-1; group D, pentoxifylline). Error bars indicate the 95% confidence interval. Pre-reperfusion value is shown at time 0. The interventions studied had no effect on platelet sequestration. Mean absolute value at time 0 = $265 \times 10^9/L$.

After a plateau phase, lasting to 240 minutes after reperfusion, neutrophils were released from the lung. In groups B, C, and D, the administration of the pharmacologic agents abolished this biphasic pattern of sequestration and subsequent release (Fig 1).

Analysis of variance showed that overall neutrophil trapping in the graft was significantly reduced in groups B, C, and D, compared with controls ($P = .026$, $P = .044$, and $P = .027$, respectively). Pentoxifylline (group D) was as effective as SIN-1 (group C) or InSP₆ (group B) in this regard ($P = .99$). There was no effect on platelet sequestration by any intervention (Fig 2).

Malonaldehyde levels were significantly higher in control lungs (group A) compared with groups B and D ($P = .02$ and $P = .001$, respectively). Pentoxifylline (group D) significantly lowered oxygen free radical activity compared with groups A, B, and C ($P = .0002$, $P = .02$, and $P < .0001$, respectively). In group C malonaldehyde levels were significantly higher than in the control group A ($P = .001$; Fig 3).

Pulmonary venous oxygen tension in the allograft lung was worst in group A. Groups B and C indicated that both inositol polyanions and SIN-1 conferred benefit with superior oxygenation achieved ($P = .0012$ and $P = .0015$, respectively), but they were similarly effective ($P > .99$). Group D was superior to all other groups

($P < .0001$; Fig 4). The difference in partial pressure of carbon dioxide between any treatment group and the control group could be due to chance ($P = .72$).

Pulmonary vascular resistance was significantly lower in all treatment groups compared with the control group A. Both SIN-1 in group C ($P = .02$) and pentoxifylline in group D ($P = .0011$) reduced the pulmonary vascular resistance compared with group B (Fig 5).

Repeated sampling did not significantly affect the hematocrit of animals in any group over the time course of the experiment.

Discussion

Our model of left single lung transplantation provides a stable and reproducible test bed, allowing fine control of ventilation and perfusion of the lung graft, while the animal is supported by the contralateral lung irrespective of the function in the newly implanted graft. The rigorous separation of the lungs in terms of sample collection ensures that there is no mixing of blood from the contralateral side.

Our data indicated that the initial 30 minutes after reperfusion is the principle time of neutrophil sequestration in the lung and is the major initiator of reperfusion injury.

The high pulmonary vascular resistance in this initial period reflects the poor compliance of the pulmonary

Fig 3. Malonaldehyde levels after reperfusion with pharmacologic interventions. Results are shown as the mean value for animals in each intervention group ($n = 5$; group A, control; group B, inositol polyanions; group C, SIN-1; group D, pentoxifylline). Error bars indicate the 95% confidence interval. Pre-reperfusion value is shown at time 0. Note that malonaldehyde levels are significantly attenuated by pentoxifylline and InSP_6 . SIN-1 administration significantly elevated free radical levels compared with controls.

vascular bed after cold ischemic storage and high potassium levels. It may also be influenced by the high levels of oxygen free radical release occurring during this period of neutrophil trapping in the lung.

As the vascular bed dilates after this initial period of reperfusion, pulmonary vascular resistance falls before rising again as a consequence of the acute lung injury resulting from reperfusion.

A period of neutrophil release was observed around this time, and the analysis of area under the curve suggests that the number of neutrophils released from the lung exceeds the number trapped there in the first 30 minutes of reperfusion. We believe the explanation lies with the pool of neutrophils remaining adherent to the vascular endothelium of the lung during retrieval. The role of these passenger leukocytes in the process of reperfusion injury is unknown.

Of the interventions studied, inositol polyanions (simple 6 carbon ring noncarbohydrate structures with multiple ester-linked phosphate or sulphate groups) appear to modulate reperfusion injury and may be of therapeutic application.⁸

Sulphated or phosphorylated carbohydrates can interact with L and P selectin and inhibit selectin-dependent

neutrophil-endothelial adhesion.¹² These observations suggest that multiple phosphate or sulphate groups may act as ligands for at least 2 of the selectins and may therefore be of use attenuating reperfusion injury.

The inhibitory effects of InSP_6 on L and P selectin are likely to be dependent on the number and structural position of phosphate groups. Inositol itself has no effect on neutrophil binding. The array of negative charge imparted by the phosphate or sulphate groups may facilitate binding to basic amino acids within the selectin molecule.

Cecconi and colleagues⁸ investigated a range of inositol polyanions. In vitro, it was clearly demonstrated that L and P selectin mediated binding to sLe^x was inhibited although no inhibition of E selectin was observed in these studies. Acute lung and peritoneal inflammation experiments indicated that tissue neutrophil accumulation was significantly reduced (by 56% in the lung) in a dose-dependent manner in those pretreated intravenously with InSP_6 . Interestingly, peripheral blood neutrophil count was unaffected by InSP_6 administration. Of the small molecules tested, InSP_6 was deemed to be the most effective and was therefore chosen for our series of experiments on lung reperfusion injury.

Fig 4. Pulmonary venous PO₂ (millimeters of mercury) with time after administration of pharmacologic interventions. Results are shown as the mean value for animals in each intervention group (n = 5; group A, control; group B, inositol polyanions; group C, SIN-1; group D, pentoxifylline). Error bars indicate the 95% confidence interval. Note superior function obtained with pentoxifylline treated lungs.

Rao and colleagues¹³ investigated the antioxidant role of myoinositol hexaphosphate (phytic acid) in a rodent model of myocardial reperfusion injury. With a Langendorff isolated heart system and a 30-minute ischemic time, treated organs showed reduced creatine kinase release and improved left ventricular function concomitant with reduced lipid peroxidation assessed by tissue malonaldehyde analysis. In contrast, Eggleton and colleagues¹⁴ noted that, although preincubation treatment of human neutrophils with InSP₆ has no effect on the basal unstimulated respiratory burst, the response to a subsequent stimulus was substantially enhanced. A substantial range of priming effect was seen, but nevertheless this work suggests a priming function in human neutrophils that may be detrimental in reperfusion injury. However, these effects may require pre-exposure to calcium and/or magnesium for the priming action to be observed *in vitro*. Our findings of attenuated reperfusion injury in the context of lung transplantation suggests that this class of drug warrants further investigation.

Nitric oxide also has beneficial effects on the pathophysiologic features of reperfusion injury and was superior to InSP₆ in our study. The mechanisms by which nitric oxide donors prevent leukocyte adhesion are probably multifactorial. Inactivation of superoxide inhibition of NADPH oxidase prevention of neutrophil

activation and effects on adhesion molecule expression may all be important. Indeed, studies using the nitric oxide synthase inhibitors L-NAME and L-NMMA to influence leukocyte adhesion to exteriorized rat mesenteric postcapillary venules suggested that adhesion increased 4-fold after nitric oxide inhibition within the first 60 minutes.¹⁵

Nitric oxide also appears to have effects on the CD11/CD18 system of adhesion molecules and reduces constitutive expression of ICAM-1 on endothelium.¹⁶ Nitric oxide, as a vasodilator, may also act through a physical mechanism by increasing pulmonary blood flow, raising shear stresses, and consequently reducing the quantity of rolling neutrophils on the pulmonary vascular endothelium.

The properties of nitric oxide with regard to endothelial protection from neutrophil adhesion and free radical generation and in attenuating platelet aggregation make nitric oxide of potential use in countering the effects of reperfusion injury.¹⁷

Some work has been performed on inhaled nitric oxide in this context, and significant pulmonary vasodilation may be achieved without systemic hypotension in concentrations of 5 to 80 ppm.¹⁸ Inhaled nitric oxide may even be of benefit when administered to the donor before organ procurement. The use of intravenously administered nitric oxide donors (typified by glyceryl

Fig 5. Pulmonary vascular resistance after reperfusion with pharmacologic interventions. Results are shown as the mean value for animals in each intervention group ($n = 5$; group A, control; group B, inositol polyanions; group C, SIN-1; group D, pentoxifylline). Error bars indicate the 95% confidence interval. All interventions significantly reduced the pulmonary vascular resistance compared with controls.

trinitrate, sodium nitroprusside, and SIN-1) is less well documented in pulmonary reperfusion injury.^{19,20} A rodent model of remote pulmonary reperfusion injury after aortic occlusion has shown intravenous nitroprusside can attenuate injury although L-NAME augments pulmonary dysfunction by what appears to be a leukocyte-dependent mechanism as judged by tissue myeloperoxidase levels.⁶

In lung transplantation some preliminary work has been performed by Naka and colleagues²¹ in a rodent model of orthotopic lung transplantation. With nitroglycerin added to the graft flush preservation fluid and a 4-hour ischemic time, after reimplantation, pulmonary hemodynamics and function were enhanced, compared with control animals. Furthermore, neutrophil sequestration, as measured by myeloperoxidase assay, suggested less accumulation in nitric oxide-treated lungs compared with controls. These studies were limited to only 30 minutes of reperfusion, and meaningful interpretation of these results is difficult.

Further work by Pinsky and colleagues⁷ confirmed that nitric oxide levels fell precipitously after reperfusion as did cyclic guanosine monophosphate. However, supplementation of the flush preservation fluid with cyclic guanosine monophosphate analog lowered pul-

monary vascular resistance and improved graft oxygenation over a 30-minute period of reperfusion. Most recently, 10 dogs underwent left lung allotransplantation by Yamashita and colleagues.¹⁹ Donor lungs were flushed with modified Euro-Collins solution and stored for 21 hours. In the study group, the donor lung received nitroprusside in the flush solution, and recipient animals received a continuous infusion during the assessment period. Superior gas exchange and hemodynamics were noted in lungs receiving nitroprusside with less myeloperoxidase activity.

King and colleagues²⁰ have experimented with an isolated rabbit lung model, using sodium nitroprusside as a nitric oxide donor, infused into the pulmonary artery. Significant improvements were observed in pulmonary hemodynamics, lung compliance, and edema, even at low dose, indicating that this may be a valuable strategy to pursue. The limitations imposed by an isolated small animal organ model and the route of infusion means that more clinically relevant large animal studies with intravenous routes of drug administration are warranted. These studies clearly suggest a potential role for nitric oxide supplementation in the attenuation of reperfusion injury.

However, concerns regarding the potential detrimen-

tal effects of nitric oxide administration have been voiced. Specifically, endogenous superoxide may combine with nitric oxide to produce the stable anion peroxynitrite and, subsequently, when protonated releases hydroxyl radicals.

It may be that a precarious balance between the beneficial effects of nitric oxide outlined earlier and the generation of toxic products may exist and that it is important to quantify these in detail if nitric oxide is to be introduced as a potential prophylactic treatment for reperfusion injury. Our study does not suggest that this is a significant problem because, although malonaldehyde levels were significantly elevated, there were no adverse effects on lung graft function.

For the purposes of this work, we selected SIN-1 as the drug of choice because it is a more efficient nitric oxide donor with few systemic effects. SIN-1 is the active metabolite of the vasodilatory drug molsidomine and is hydrolyzed to form SIN-1A, which undergoes oxygen-dependent release of nitric oxide and superoxide. Rat studies on myocardial muscle have suggested that this agent is more effective in preventing leukocyte adhesion and transmigration compared with glyceryl trinitrate and sodium nitroprusside and is more efficient at preventing platelet aggregation. However, SIN-1 is known to spontaneously decompose to release superoxide radicals and nitric oxide and to inhibit release of plasminogen activator inhibitor from platelets.

The addition of pentoxifylline in the recipient confers significant additional benefit over the other interventions studied. Pentoxifylline has many functions, in particular inhibiting the adherence of neutrophils to endothelium and preventing leukocyte degranulation. It may also inhibit the important interleukin-1, and tumor necrosis factor, which are critical in the pathogenesis of ischemia-reperfusion injury. Pentoxifylline clearly improves and augments function by reducing neutrophil trapping in the lung and subsequent free radical release. This agent has been beneficial in previous porcine models of lung transplantation but never before compared with other potential therapies. There has been some suggestion that pentoxifylline is effective when administered in flush solutions but not in recipient alone.²² Our findings seem to support the former, although results are difficult to compare between different dose regimens and models.

These simple pharmacologic interventions can be instituted easily in the operating theater in human recipients. Our studies suggest that all the agents studied are free of systemic adverse effects.

Pentoxifylline (already licensed for use in humans in peripheral vascular disease) is an inexpensive and safe

drug for use in lung transplant recipients and appears to be superior to inositol polyanions and SIN-1 in terms of graft function through attenuation of neutrophil sequestration and free radical release. Adoption of these interventions may lead to considerable improvements in death and morbidity, shorter intensive care stays, and costs among lung transplant recipients.

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