Superoxide anion scavengers restore NO-mediated pulmonary vasodilation after lung transplantation

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Seki, Sumihiko, Nicholas A. Flavahan, Nicholas G. Smedira, and Paul A. Murray. Superoxide anion scavengers restore NO-mediated pulmonary vasodilation after lung transplantation. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H42–H46, 1999.—Left lung autotransplantation (LLA) results in a chronic attenuation in endothelium-dependent, nitric oxide (NO)-mediated pulmonary vasodilation. We tested the hypothesis that this abnormality involves a decrease in the effective concentration of NO due to inactivation by superoxide anion. Size- and position-matched pulmonary arterial rings were isolated from the right (control) and left (LLA) lungs of seven dogs 1–5 mo post-LLA. The rings were suspended for isometric tension recording and contracted with phenylphrine, and cumulative dose-response curves for acetylcholine; calcium ionophore A-23187; superoxide dismutase; endothelial xanthine oxidase. In the pulmonary circulation; endothelium-dependent vasodilators; acetylcholine; calcium ionophore A-23187; superoxide dismutase; tiron; oxypurinol.

We have previously demonstrated that LLA results in an impairment in endothelium-dependent, nitric oxide-mediated pulmonary vasodilation in conscious dogs (18) and in isolated canine pulmonary arterial rings (5). The aim of the present study was to investigate the cellular mechanism responsible for the attenuation in nitric oxide-mediated pulmonary vasodilation post-LLA. Our hypothesis was that this mechanism would involve a decrease in the effective concentration of nitric oxide due to inactivation by superoxide anion. To test this hypothesis, we investigated the effects of the following on the pulmonary vasorelaxant responses to the endothelial agonists ACh and calcium ionophore (A-23187) post-LLA: 4,5-dihydroxy-1,3-benzenedisulfonic acid (tiron), a low-molecular-weight superoxide anion scavenger that can enter cells (3); superoxide dismutase (SOD), which scavenges superoxide anion in the extracellular space; and oxypurinol, a xanthine oxidase inhibitor.

MATERIALS AND METHODS

All surgical procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee.

Surgical preparation. The surgical procedure for LLA has been previously described in detail (13). Microfilaria-free male mongrel dogs were premedicated with morphine sulfate (10 mg im) and anesthetized with pentobarbital sodium (20 mg/kg iv) and fentanyl citrate (15 µg/kg iv). After tracheal intubation, the lungs were mechanically ventilated. Anesthesia was maintained with halothane (~1.2%, end tidal). A left thoracotomy was performed via the fifth intercostal space using sterile surgical technique, and the pericardium was incised ventral to the phrenic nerve. LLA was achieved by serial anastomoses of the left pulmonary veins, left main pulmonary artery, and left mainstem bronchus. Heparin (100 U/kg) was administered intravenously just before the LLA procedure. The inferior, middle, and superior left pulmonary veins were dissected to their point of confluence with the left atrium, cross-clamped, divided, and anastomosed as a patch to the left atrial appendage. The left main bronchus was then clamped just distal to the carina, divided, and anastomosed. The left main pulmonary artery was dissected free of connective tissue, cross-clamped, divided, and anastomosed. The entire LLA procedure took 2–3 h, but the total left pulmonary artery cross-clamp time was only 10–20 min. Morphine sulfate (10 mg im) was administered postoperatively for pain as required. Ampicillin (1 g iv), cefazolin (1 g iv), and gentamicin (80 mg iv) were administered intraoperatively and postoperatively for 10 days.

Organ chamber experiments. One to five months after LLA, seven otherwise healthy dogs, weighing 24–32 kg, were anesthetized with pentobarbital sodium (20 mg/kg iv) and...
fentanyl citrate (15 µg/kg iv), exsanguinated by controlled hemorrhage, and euthanized with a bolus of saturated KCl injected intravenously. After performing a left lateral thoracotomy, we removed the heart and lungs en bloc. Using sterile technique, we dissected right (control) and left (LLA) intralobar pulmonary arteries (2–4 mm, ID) free and immersed them in cold modified Krebs-Ringer bicarbonate solution of the following composition (in mM): 118.3 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 25.0 NaHCO3, 0.016 Ca-EDTA, and 11.1 glucose. The arteries were cut into 0.5-cm length rings, with care taken not to damage the endothelium. The rings were suspended between two stainless steel stirrups in organ chambers filled with 25 ml modified Krebs-Ringer bicarbonate solution (37°C) and gassed with 95% O2-5% CO2. One of the stirrups was anchored, and the other was connected to a strain gauge (Grass model FT03 force displacement transducer) for the measurement of isometric tension (Gould 3000S). The rings from the same relative anatomic locations in the right and left lungs were used as paired rings.

Experimental protocols. Pulmonary arterial rings were stretched at 10-min intervals, in increments of 0.5 g, to reach optimal resting tone. Optimal resting tone was the minimum amount of stretch required to achieve the largest contractile response to KCl (20 mM) and was determined in preliminary experiments to be 5 g for the size of arteries used in these studies (2–4 mm, ID). After the arterial rings had been stretched to their optimal resting tone, the contractile response to 60 mM KCl was measured. After washout of KCl from the organ chamber and the return of isometric tension to prestimulation values, a concentration-effect curve for the sympathetic α-adrenoreceptor agonist phenylephrine was obtained for each ring by increasing the concentration of phenylephrine in half-log increments (from 10−8 to 3 × 10−5 M) after the response to each preceding concentration had reached a steady state. Initial experiments showed that phenylephrine caused β-adrenergic relaxation, in addition to α-adrenergic contraction, in these arteries. Thus the rings were pretreated with the β-adrenergic antagonist propranolol (5 × 10−6 M, incubated for 30 min) before treatment with phenylephrine for all protocols. After washout and the return of isometric tension to baseline values, rings were pretreated for 30 min with one of the following drugs: tiron (10 mM), an extra- and intracellular superoxide anion scavenger; SOD (150 U/ml), an extracellular superoxide anion scavenger; oxypurinol (10−4 M), a xanthine oxidase inhibitor; or no drug. The rings were precontracted to 50% of the maximal contractile response to phenylephrine (ED50), and then ACh (10−9 to 10−6 M) or A-23187 (10−9 to 10−6 M) was cumulatively administered. Only one vasorelaxant concentration-effect curve was performed in each ring.

Drugs and solutions. A-23187, ACh chloride, phenylephrine HCl, propranolol, oxypurinol, SOD (from canine erythrocytes), and tiron were obtained from Sigma Chemical (St. Louis, MO). All concentrations are expressed as the final molar concentration in the organ chamber. Stock solutions were prepared each day. A stock solution of A-23187 was prepared using dimethyl sulfoxide (final organ chamber concentration, 0.00004–0.013% vol/vol). Oxypurinol was dissolved in NaOH and diluted in distilled water. Tiron was dissolved directly in the modified Krebs-Ringer bicarbonate solution. All other drugs were dissolved and diluted in distilled water. The vehicles have no effect on isometric tension at the concentrations used in this study (5).

Data analysis. Values are presented as means ± SE. Responses to the vasorelaxants are expressed as a percentage of the contraction to phenylephrine. Experiments were performed on two dogs at 1 mo post-LLA, four dogs at 2–3 mo post-LLA, and one dog at 5 mo post-LLA. Results from all dogs have been combined because there are no apparent differences in responses to the agonists with respect to time post-LLA (5). The inhibitory concentrations (IC) of A-23187 that caused 50% relaxation of the contraction to phenylephrine (IC50) were interpolated from the linear portion of the concentration-effect curves by regression analysis and are presented as log IC50 values. Maximal responses to ACh are expressed as percent relaxation of the contractile response to phenylephrine. Because LLA reduced the maximal response to ACh, IC50 values were not calculated for this agonist. Student’s t-test for paired samples was used to compare the log IC50 values and the maximal responses. Values were considered to be significant at P < 0.05.

RESULTS

The pulmonary vasorelaxant responses to ACh and A-23187 in untreated (no drug) control and LLA rings are summarized in Fig. 1. Compared with control rings, LLA caused a downward shift in the ACh concentration-effect curve, resulting in a decrease (P < 0.01) in the maximal vasorelaxant response from 88 ± 5 to 63 ± 5% (Fig. 1A). The dose-response relationship for A-23187 was also inhibited post-LLA, with the concentration-effect curve shifted (P < 0.01) to the right in LLA rings (log IC50 = −7.12 ± 0.09) compared with control rings (log IC50 = −7.59 ± 0.09), and with no change in the maximal response to A-23187 (Fig. 1B).

Fig. 1. Pulmonary vasorelaxant responses to ACh (A) and calcium ionophore A-23187 (B) in pulmonary arterial rings isolated from right (control) and left [left lung autotransplantation (LLA)] lungs without pretreatment (no drugs). Relaxations are expressed as percentage of phenylephrine (PE) precontraction and are presented as means ± SE.
Control and LLA rings were pretreated with the extra- and intracellular superoxide anion scavenger tiron. Tiron had no effect on baseline tension. The pulmonary vasorelaxant responses to ACh and A-23187 in control and LLA rings after pretreatment with tiron are summarized in Fig. 2. Under these conditions, the ACh dose-response relationship was similar in control and LLA rings, with maximal vasorelaxant responses of 86 ± 7 and 75 ± 10%, respectively (Fig. 2A). After pretreatment with tiron, the pulmonary vasorelaxant response to A-23187 in LLA rings (log IC50 = −7.21 ± 0.13) was similar to the response measured in control rings (log IC50 = −7.27 ± 0.09), as summarized in Fig. 2B.

Control and LLA rings were also pretreated with the extracellular superoxide anion scavenger SOD. SOD had no effect on baseline tension. The pulmonary vasorelaxant responses to ACh and A-23187 in control and LLA rings after pretreatment with SOD are summarized in Fig. 3. Under these conditions, the ACh dose-response relationship was similar in control and LLA rings, with maximal vasorelaxant responses of 89 ± 5 and 83 ± 5%, respectively (Fig. 3A). The pulmonary vasorelaxant response to A-23187 was also restored after pretreatment with SOD in LLA rings (log IC50 = −7.39 ± 0.11) compared with control rings (log IC50 = −7.37 ± 0.17), as summarized in Fig. 3B.

To identify the source of superoxide anion production, control and LLA rings were pretreated with the xanthine oxidase inhibitor oxypurinol. Oxypurinol had no effect on baseline tension. The pulmonary vasorelaxant responses to ACh and A-23187 in control and LLA rings after pretreatment with oxypurinol are summarized in Fig. 4. Under these conditions, the ACh dose-response relationship was similar in control and LLA rings, with maximal vasorelaxant responses of 86 ± 7 and 87 ± 9%, respectively (Fig. 4A). Oxypurinol also restored the pulmonary vasorelaxant response to A-23187 in LLA rings (log IC50 = −7.28 ± 0.13) compared with control rings (log IC50 = −7.36 ± 0.11), as summarized in Fig. 4B.

**DISCUSSION**

LLA is characterized by chronic changes in pulmonary vasoregulation. An impairment in endothelium-dependent, nitric oxide-mediated pulmonary vasodilation post-LLA was demonstrated in both our in vivo (18) and in vitro (5) studies. The goal of the present in vitro study was to identify the cellular mechanism responsible for this defect after LLA. Our results indicate that the attenuated response to nitric oxide-mediated pulmonary vasodilation post-LLA involves inactivation of nitric oxide by superoxide anion. Moreover, endothelial xanthine oxidase appears to be an important source of superoxide anion production post-LLA.

In in vivo studies of chronically instrumented, conscious dogs, we have previously observed that LLA results in a chronic increase in pulmonary vascular
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Left (LLA) lungs after pretreatment with oxypurinol (10 μM) (B). Because of its low molecular weight, tiron can inactivate superoxide anion in both the extracellular and intracellular spaces. Tiron restored the pulmonary vasorelaxant responses to ACh and A-23187, which demonstrates that superoxide anion is involved in the attenuated responses to these endothelial activators post-LLA. SOD also restored the attenuated responses to ACh and A-23187. Because SOD probably does not enter cells (22), these results suggest that inactivation of nitric oxide by superoxide anion can occur in the extracellular space. Finally, we investigated whether endothelial cells were the source of superoxide anion. It is likely that there are multiple pathways for the production of superoxide anions in endothelial cells. We utilized oxypurinol to specifically assess the role of endothelial xanthine oxidase in the generation of superoxide anion post-LLA. Oxypurinol, which inhibits xanthine oxidase (9), completely restored the pulmonary vasorelaxant responses to ACh and A-23187 post-LLA. These results suggest that endothelial xanthine oxidase production of superoxide anion is the cellular mechanism responsible for the attenuated response to nitric oxide-mediated vasorelaxants post-LLA.

The mechanism responsible for increased activity of endothelial xanthine oxidase post-LLA is unknown. Ischemia-reperfusion injury during the LLA surgical procedure could be responsible. A closed-chest hypothermic cardiopulmonary bypass (CPB) of 2.5-h duration results in pulmonary vascular hyperreactivity up to 14 days post-CPB (20) and causes a selective defect in the pulmonary vasodilator response to ACh 3–4 days post-CPB (26). In the present study, the total ischemia time (left pulmonary artery cross-clamp time) for the LLA procedure was only 10–20 min. Moreover, experiments were performed 1–5 mo post-LLA. Thus it does not seem likely that a chronic increase in endothelial xanthine oxidase activity post-LLA would be caused by ischemia-reperfusion injury during surgery. Although speculative, it is possible that the chronic increase in pulmonary vascular resistance post-LLA (13), or changes in pulmonary artery blood flow patterns (e.g., turbulent flow), could lead to endothelial dysfunction and the increased production of superoxide anion.

In summary, the pulmonary vasorelaxant responses to ACh and A-23187 were attenuated post-LLA compared with control. This attenuating effect was entirely reversed by pretreatment with the superoxide anion scavengers tiron and SOD, or the xanthine oxidase inhibitor oxypurinol. These results strongly support the theory that the attenuated response to nitric oxide-mediated pulmonary vasodilation post-LLA involves inactivation of nitric oxide by superoxide anion generated by endothelial xanthine oxidase. Abnormal superoxide anion production could play a role in the development of chronic vasculopathy posttransplantation.

The authors thank Steve Schomisch, Pantelis Konstantinopulos, Mike Trentanelli, George Markakis, and James Palazzolo for technical work and Ronnie Sanders for secretarial support in preparing the manuscript.

This study was supported by National Heart, Lung, and Blood Institute Grants HL-40361, HL-38291, and HL-56091. S. Seki was also supported by Professor Akiyoshi Namiki, Dept. of Anesthesiology, Sapporo Medical Univ. School of Medicine, Sapporo, Japan.
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