Endothelial defect mediates attenuated vasorelaxant response to isoproterenol after lung transplantation

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Yoshida, Kenichi, Nicholas A. Flavahan, Mayumi Horibe, Nicholas G. Smedira, and Paul A. Murray. Endothelial defect mediates attenuated vasorelaxant response to isoproterenol after lung transplantation. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H159–H166, 1999.—We have previously demonstrated that pulmonary vasodilation in response to isoproterenol is attenuated in conscious dogs after left lung autotransplantation (LLA). Our present goal was to identify the cellular mechanism responsible for this dysfunction. Size- and position-matched pulmonary arterial rings were isolated from the right (control) and left (LLA) lungs of 23 dogs 1–14 mo post-LLA. The rings were suspended for isometric tension recording and precontracted, and the vasorelaxant responses to activators of the β-adrenoceptor signaling pathway were examined. With the endothelium intact the maximal pulmonary vasorelaxant response to isoproterenol was reduced (P < 0.02) to 57 ± 9% in LLA rings, compared with 87 ± 3% in control rings. Responses to the Gs protein activator cholera toxin were also attenuated post-LLA, with the concentration-effect curve shifted to the right (P < 0.01) and no change in the maximal response. In contrast, the vasorelaxant responses to forskolin (adenyl cyclase activator) or dibutyryl cAMP were similar in endothelium-intact control and LLA rings. In endothelium-denuded rings the maximal vasorelaxant responses to isoproterenol were reduced (P < 0.01) to ~25% in both control and LLA rings. In denuded rings cholera toxin, forskolin, and dibutyryl cAMP caused 100% vasorelaxation, and the IC50 values for these agonists were similar in control and LLA rings. Isoproterenol increased (P < 0.05) tissue cAMP to the same extent in control and LLA rings with or without endothelium. In contrast, isoproterenol increased (P < 0.05) tissue cGMP only in endothelium-intact rings, and this effect was reduced (P < 0.05) ~50% in LLA rings compared with control. Oxypurinol (endothelial xanthine oxidase inhibitor) restored the pulmonary vasorelaxant response to isoproterenol in endothelium-intact LLA rings. Our results provide the first evidence that activation of the β-adrenoceptor signaling pathway in endothelium-intact pulmonary arterial rings results in an increase in cGMP. Moreover, the attenuation in β-adrenoceptor-mediated pulmonary vasorelaxation post-LLA is due to inactivation of nitric oxide by endothelium-derived superoxide anion.

pulmonary circulation; cholera toxin; forskolin; dibutyryl adenosine 3’,5’-cyclic monophosphate; β-adrenoceptor agonist; cyclic nucleotides

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 described previously in detail (18). Briefly, conditioned (microfilaria-free) mongrel dogs (20–30 kg) were premedicated with morphine sulfate (10 mg im) and anesthetized with pentobarbital sodium (20 mg/kg iv) and fentanyl citrate (15 µg/kg iv). Tracheal intubation was performed, and the lungs were mechanically ventilated. Anesthesia was maintained with halothane (~1.2% end tidal). A left thoracotomy was performed through the fifth intercostal space using sterile surgical technique. LLA was achieved by serial section and anastomoses of the left pulmonary veins, left main pulmonary artery, and left main stem bronchus. Morphine sulfate was administered for postoperative analgesia as required. Intravenous antibiotics (ampicillin, cefazolin, and gentamicin) were administered for 10 days postoperatively.
Organ Chamber Experiments

From 1 to 14 mo after LLA, 23 otherwise healthy dogs were anesthetized with pentobarbital sodium (20 mg/kg iv) and fentanyl citrate (15 µg/kg iv) and placed on positive pressure ventilation. The mobilizable blood volume was removed by controlled hemorrhage, and the dogs were killed with a bolus of saturated KCl injected intravenously. A left lateral thoracotomy was performed, and the heart and lungs were removed en bloc. Under sterile conditions right (control) and left (LLA) intralobar pulmonary arteries (2–4 mm ID) were dissected free and immersed 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 cleaned of loose connective tissue and cut into 0.5-cm-length rings, with special care taken not to touch the luminal surface. In some arterial rings the endothelium was intentionally removed by inserting forceps tips into the lumen and rolling the rings over damp filter paper. Removal of the endothelium was later verified by assessing the vasorelaxant response to acetylcholine (10−6 M). Endothelial denudation reduced the vasorelaxant response to acetylcholine from 93 ± 7 to 7 ± 4%. The rings were mounted horizontally between two stainless steel stirrups in organ chambers filled with 25 ml of modified Krebs-Ringer bicarbonate solution (37°C), gassed with 95% O2-5% CO2. One of the stirrups was anchored and the other was connected to a strain gauge (Grass model FT03) for the measurement of isometric tension (Gould 3000S). Rings with and without endothelium 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 level of stretch required to achieve the largest contractile response to 20 mM KCl 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 removal of KCl from the organ chambers and the return of isometric tension to prestimulation values, a concentration-effect curve for the sympathetic α1-adrenergic receptor agonist phenylephrine was obtained by increasing the concentration of the agonist in half-log increments (10−8 to 3 × 10−5 M) after the response to each preceding concentration had reached a steady state.

Protocol 1. This experimental series tested the hypothesis that the pulmonary vasorelaxant response to the sympathetic β-adrenergic receptor agonist isoproterenol would be attenuated post-LLA. Control and LLA rings with and without endothelium were pretreated (30 min) with the selective sympathetic α1-adrenergic antagonist prazosin (10−7 M) to inhibit the α1-agonist activity of isoproterenol. The rings were then precontracted with angiotensin II (10−9 M) to a level of tension equivalent to 50% of the maximal contractile response (ED50) to phenylephrine, followed by the cumulative administration of isoproterenol (10−8 to 3 × 10−5 M).

Protocol 2. This experimental series tested the hypothesis that the attenuated response to isoproterenol post-LLA was due to an endothelial defect involving G protein. Control and LLA rings with and without endothelium were precontracted to the ED50 level of tension with phenylephrine, followed by the cumulative administration of the Gs activator, cholera toxin (0.001–0.3 mg/). 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 phenylephrine for all protocols.

Protocol 3. This experimental series tested the hypothesis that the attenuated response to isoproterenol post-LLA was due to an endothelial defect primarily involving adenylyl cyclase. Control and LLA rings with and without endothelium were precontracted to the ED50 level of tension with phenylephrine, followed by the cumulative administration of the adenylyl cyclase activator forskolin (10−9 to 10−5 M).

Protocol 4. This experimental series tested the hypothesis that the attenuated response to isoproterenol post-LLA was due to an endothelial defect in the signal transduction pathway distal to cAMP phosphodiesterase activity. Control and LLA rings with and without endothelium were precontracted to the ED50 level of tension with phenylephrine, followed by the cumulative administration of dibutyryl cAMP (10−6 to 10−4 M), a membrane permeable analog of cAMP that bypasses adenylyl cyclase and is not metabolized by cAMP phosphodiesterase.

Protocol 5. This experimental series tested the hypothesis that the attenuated response to isoproterenol was due to an endothelial defect involving the cGMP signal transduction pathway. Pulmonary arterial rings with and without endothelium were prepared. The adequacy of endothelial denudation was tested with acetylcholine (10−6 M). The rings were allowed to equilibrate for 90 min without tension. A single concentration of phenylephrine (10−6 M) was added to the organ chamber. The rings were placed in liquid nitrogen at either time 0 (control) or after 60 s of exposure to one of the agonists. Preliminary time-course experiments demonstrated that a 60 s exposure to these agonists resulted in maximal increases in cAMP and cGMP. The frozen samples were stored at −80°C. The rings were homogenized in 6% trichloroacetic acid and 55 µM theophylline, and stored for 24 h at 4°C. Precipitated protein was separated from the soluble extract by centrifugation (2,500 g) for 15 min at 4°C. Trichloroacetic acid was removed from the sample with four successive water-saturated ether extractions. The samples were then evaporated at 70°C, gassed with N2, and stored at −20°C. Before analysis, each sample was resuspended in 1 ml of sodium acetate buffer (0.05 M, pH 6.2) and divided into two aliquots for simultaneous measurement of both cAMP and cGMP. The concentrations of cAMP and cGMP in the tissue extracts were determined after acetylation using DuPont cAMP and cGMP radioimmunoassay kits (NEK-033, NEX-133). Precipitated protein was digested in sodium hydroxide (2 N) for 1 h at 50°C and then diluted to 0.5 N. The digest was assayed for protein using a bicinchoninic acid protein assay kit (BCA Protein Assay Reagent, Pierce, Rockford, IL). Protein content was calculated from standards prepared from bovine serum albumin. Successive dilutions of bovine serum albumin were made in sodium hydroxide (0.5 N).

Protocol 6. This experimental series tested the hypothesis that inactivation of nitric oxide by endothelium-derived superoxide anion mediated the attenuated pulmonary vasorelaxant response to isoproterenol post-LLA. Control and LLA rings with endothelium were pretreated with prazosin (10−7 M) and oxyurinol (10−4 M), an inhibitor of endothelial xanthine oxidase. The rings were precontracted with angioten-
isoproterenol (10^-9 M), followed by the cumulative administration of isoproterenol (10^-8 to 3 x 10^-5 M).

Drugs and Solutions

Acetylcholine chloride, cholea toxin, dibutryl cAMP, forskolin, l-isoproterenol bitartrate, oxyipurinol, phenylephrine HCl, and prazosin HCl were obtained from Sigma Chemical (St. Louis, MO). All drugs were of the highest purity commercially available. All concentrations are expressed as the final concentration in the organ chamber. Stock solutions were prepared on the day of the experiment. Forskolin was prepared as 10^-2 M stock solution in 70% ethanol and diluted in distilled water. Oxyipurinol was dissolved in NaOH and diluted in distilled water. All other drugs were dissolved and diluted in distilled water. At the concentrations used in this study, the vehicles had no effect on isometric tension (4).

Data Analysis

Values are expressed as means ± SE. Vasorelaxant responses of the agonists are expressed as the percentage of the contraction to either angiotensin II (protocols 1 and 6) or phenylephrine (protocols 2–4). Experiments were performed on 4 dogs at 1 mo post-LLA, 11 dogs at 2–3 mo post-LLA, 4 dogs at 4–5 mo post-LLA, 1 dog at 8 mo post-LLA, and 3 dogs at 13–14 mo post-LLA. Results from all dogs have been combined because there were no apparent differences in responses to the agonists with respect to time post-LLA. The inhibitory concentrations of the agonists causing 50% relaxation of the contraction to either angiotensin II or phenylephrine were interpolated from the linear portion of the concentration-effect curve by regression analysis and are presented as log IC50 values. Student’s t-test for paired samples was used to compare the log values. When more than two means were compared, analysis of variance was used. If a significant F value was found, Bonferroni and Dunn post hoc tests were employed to identify differences between groups. Values were considered to be significant at P < 0.05.

RESULTS

Effect of LLA on Pulmonary Vasorelaxant Response to Isoproterenol

To assess the vasorelaxant response to isoproterenol, control and LLA rings were pretreated with prazosin (10^-7 M) and precontracted with angiotensin II (10^-9 M) rather than phenylephrine, because isoproterenol has α1-agonist activity at higher concentrations and because LLA potentiates the pulmonary vasosconstrictor response to α1-adrenoceptor activation (Fig. 1). In control rings with the endothelium intact, isoproterenol caused dose-dependent relaxation reaching a maximum of 87 ± 3% (Fig. 2A). The relaxation response to isoproterenol was attenuated in LLA rings, with the concentration-effect curve shifted downward and the maximum response to the β-agonist reduced (P < 0.02) to 57 ± 9% (Fig. 2A). In contrast, in endothelium-denuded rings the relaxation responses to isoproterenol were similar in control and LLA rings (Fig. 2B). However, the relaxation responses to isoproterenol were reduced (P < 0.01) in endothelium-denuded control and LLA rings compared with endothelium intact rings, with maximum relaxation responses reaching only 25 ± 4 and 22 ± 4%, respectively. These results indicate that LLA attenuates the endothelium-dependent component of isoproterenol-induced pulmonary vasorelaxation.

Effect of LLA on Pulmonary Vasorelaxant Response to Cholera Toxin

To assess the vasorelaxant response to cholera toxin, control and LLA rings were precontracted to 50% of their maximal response to phenylephrine (ED50). Cholera toxin induced concentration-dependent vasorelaxation in both endothelium-intact and endothelium-denuded rings (Fig. 3). In endothelium-intact rings LLA caused a rightward shift in the concentration-effect curve for cholera toxin without changing the maximal response (Fig. 3A). The IC50 (log mg/l) for cholera toxin (Table 1) was increased (P < 0.01) post-LLA (1.50 ± 0.09) compared with control (1.46 ± 0.1). In contrast, in endothelium-denuded rings the vasorelaxant response to cholera toxin was attenuated (P < 0.05) in endothelium-denuded control rings but not in endothelium-denuded LLA rings (Fig. 3 and Table 1).
Effect of LLA on Pulmonary Vasorelaxant Response to Forskolin

After precontraction with phenylephrine, forskolin induced concentration-dependent vasorelaxation in both endothelium-intact and endothelium-denuded rings (Fig. 4). Compared with control rings, the vasorelaxant response to forskolin was not altered post-LLA in either endothelium-intact or endothelium-denuded rings (Fig. 4). Compared with endothelium-intact rings, the vasorelaxant responses to forskolin were attenuated ($P < 0.05$) in endothelium-denuded control and LLA rings (Table 1).

Effect of LLA on cAMP Accumulation Induced by Isoproterenol, Cholera Toxin, and Forskolin

There were no significant differences in baseline cAMP values between control and LLA rings, or between endothelium-intact and endothelium-denuded rings (Fig. 6). Phenylephrine (without propranolol pretreatment) modestly increased ($P < 0.05$) cAMP to the same extent in control and LLA rings (Fig. 6). The increases ($P < 0.05$) in cAMP in response to isoproterenol, cholera toxin, and forskolin were also similar in control and LLA rings with or without endothelium (Fig. 6).

Effect of LLA on cGMP Accumulation Induced by Isoproterenol, Cholera Toxin, and Forskolin

Endothelial denudation slightly decreased ($P < 0.05$) baseline cGMP values in control and LLA rings (Fig. 7). Phenylephrine increased ($P < 0.05$) cGMP in endothelium-intact control and LLA rings (Fig. 7). Isoproterenol, cholera toxin, and forskolin only increased ($P < 0.05$) in both control and LLA rings with or without endothelium (Fig. 7).
The overall goal of this in vitro study was to assess the extent to which LLA altered the pulmonary vasodilator response to sympathetic β-adrenoceptor activation and to identify the cellular mechanism responsible for any observed defect.

Classically, vascular smooth muscle has been considered to be the key component in the signal transduction pathway mediating vasodilation in response to β-adrenoceptor activation (11). The coupling of the β-adrenoceptor to adenyl cyclase is regulated by a family of heterotrimeric proteins known as the stimulatory GTP binding proteins (Gs). Activation of adenyl cyclase increases the production of cAMP. The cellular mechanism that mediates cAMP-induced vasorelaxation has not been completely elucidated but appears to involve a decrease in intracellular calcium concentration, a reduction in myosin light chain kinase activity, and subsequent dephosphorylation of myosin light chain (16). In the present study, isoproterenol, cholera toxin, forskolin, and dibutyryl cAMP each caused concentration-dependent relaxation that was associated with the accumulation of cAMP. The vasorelaxation and the accumulation of cAMP in response to these agents were similar in control and LLA arteries. These results indicate that the β-adrenergic signaling pathway is normal in the vascular smooth muscle of LLA arteries. In control and LLA rings, relaxation to isoproterenol was greater in endothelium-intact compared with endothelium-denuded rings. Furthermore, isoproterenol-induced relaxation was only reduced in endothelium-intact LLA rings compared with control rings. These results suggest that isoproterenol-induced pulmonary vasorelaxation involves both an endothelium-dependent and a vascular smooth muscle component. LLA attenuates β-adrenoceptor-mediated relaxation by inhibiting the endothelium-dependent component of the response.

Table 1. Effects of LLA and endothelial denudation on pulmonary vasorelaxation

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Endothelium</th>
<th>log IC₅₀</th>
<th>Maximal Response, %</th>
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<tbody>
<tr>
<td>Isoproterenol</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>8</td>
<td>+</td>
<td>0.16</td>
<td>101.4 ± 2.3</td>
</tr>
<tr>
<td>LLA</td>
<td>8</td>
<td>+</td>
<td>0.18</td>
<td>98.4 ± 2.4</td>
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<tr>
<td>Cholera toxin</td>
<td></td>
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<tr>
<td>Control</td>
<td>8</td>
<td>+</td>
<td>0.14</td>
<td>101.7 ± 2.7</td>
</tr>
<tr>
<td>LLA</td>
<td>8</td>
<td>+</td>
<td>0.15</td>
<td>99.2 ± 2.5</td>
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<tr>
<td>Forskolin</td>
<td></td>
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<tr>
<td>Control</td>
<td>8</td>
<td>+</td>
<td>0.16</td>
<td>101.4 ± 2.3</td>
</tr>
<tr>
<td>LLA</td>
<td>8</td>
<td>+</td>
<td>0.16</td>
<td>99.2 ± 2.5</td>
</tr>
<tr>
<td>Dibutyryl cAMP</td>
<td></td>
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<tr>
<td>Control</td>
<td>8</td>
<td>+</td>
<td>0.17</td>
<td>101.7 ± 2.7</td>
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<tr>
<td>LLA</td>
<td>8</td>
<td>+</td>
<td>0.17</td>
<td>99.2 ± 2.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = number of dogs. IC₅₀, concentration (cholera toxin mg/l, other agonists M) of agonist causing 50% relaxation of precontraction. Maximal response is %relaxation of precontraction. Because left lung autotransplantation (LLA) reduced relaxation of precontraction. Maximal response is %relaxation of precontraction. IC₅₀ values were not calculated for this agonist.*P < 0.05 LLA vs. control. †P < 0.05 endothelium intact (+) vs. endothelium denuded (−).

Fig. 4. Pulmonary vasorelaxant response to forskolin in pulmonary arterial rings isolated from right (control) and left (LLA) lungs with endothelium intact (A) or with endothelium removed (B). Relaxations are expressed as %precontraction. Values are means ± SE.
Previous studies have suggested that there is an endothelial component to β-adrenoceptor-mediated and cAMP-dependent vasodilation (7, 8). Autoradiographic studies have demonstrated that β-adrenoceptors are present on vascular endothelial cells (17, 27), and endothelial denudation has been shown to attenuate the vasorelaxant response to isoproterenol (7, 8, 20). Although the cellular mechanism responsible for endothelium-dependent vasodilation in response to β-adrenoceptor activation has not been fully elucidated, there is evidence to suggest a role for nitric oxide, as well as a possible synergistic interaction between cGMP- and cAMP-mediated vasoregulation, at the level of the smooth muscle (8, 26). This latter effect is thought to be due to cGMP-mediated inhibition of cAMP phosphodiesterase (PDE III) (2, 13, 15). It has been postulated that the basal production of endothelium-derived nitric oxide can amplify the direct vascular smooth muscle relaxation response to cAMP-dependent vasodilators (26). We have recently reported that although there is a synergistic interaction between cAMP- and cGMP-dependent mechanisms in pulmonary artery, this synergy requires activation of KATP channels (6). In the present study, endothelium denudation in control rings markedly reduced the vasorelaxant response to isoproterenol, moderately reduced responses to cholera toxin and forskolin, and slightly decreased the response to dibutyryl cAMP. To determine whether this endothelial modulation reflected a synergistic interaction between cGMP- and cAMP-mediated vasorelaxation, or whether it involved an increase in nitric oxide-stimulated cGMP accumulation, the effects of the agonists on cAMP and cGMP levels were measured in endothelium-denuded and endothelium-intact rings. Isoproterenol, cholera toxin, and forskolin each increased cGMP in endothelium-containing rings, and this effect was abolished by endothelium denudation. In contrast, endothelium denudation did not significantly alter the increases in cAMP in response to these agonists. These results suggest that these agonists activated pulmonary artery endothelial cells to produce nitric oxide, which then increased cGMP and enhanced the vasorelaxant responses. Differences in the extent of endothelial modulation likely reflects variable potencies of the agonists to induce endothelium-dependent and -independent components of relaxation.

LLA attenuated the endothelium-dependent component, but not the endothelium-independent component, of relaxation to isoproterenol and to cholera toxin. The

![Graph A](image1)

**Fig. 5.** Pulmonary vasorelaxant response to dibutyryl cAMP in pulmonary arterial rings isolated from right (control) and left (LLA) lungs with endothelium intact (A) or with endothelium removed (B). Relaxations are expressed as %precontraction. Values are means ± SE.

![Graph B](image2)

**Fig. 6.** Compared with baseline (BL) values, increases in cAMP in response to phenylephrine (Phe), isoproterenol (Iso), cholera toxin (CTx), and forskolin (Forsk) were similar in control and LLA rings with (A) or without (B) endothelium. Values are means ± SE.
ability of these agonists to increase vascular smooth muscle cGMP was also decreased in LLA compared with control rings, which suggests that the activity of nitric oxide induced by activation of the \( \beta \)-adrenoreceptor-Gs protein signaling pathway is reduced post-LLA. However, in contrast to isoproterenol and cholera toxin, LLA did not inhibit the endothelial component of the vasorelaxant response to either forskolin or dibutyryl cAMP, and it did not inhibit the ability of forskolin to increase the levels of cGMP. These results suggest that the LLA-induced dysfunction in this endothelial signaling pathway is localized to the Gs protein and does not affect the signaling pathway distal to adenylyl cyclase.

LLA-induced impairment of endothelium-dependent relaxation to acetylcholine and A-23187 is due to inactivation of nitric oxide by superoxide anion derived from endothelial xanthine oxidase (25). In the present study the xanthine oxidase inhibitor oxypurinol also prevented the impairment in relaxation to isoproterenol induced by LLA. These results suggest that LLA causes a generalized decrease in endothelium-dependent relaxation by increasing the activity of xanthine oxidase. The inability of LLA to diminish endothelial responses to forskolin or dibutyryl cAMP may reflect differential regulation of xanthine oxidase by the \( \beta \)-adrenergic cAMP signaling system in endothelial cells.

Just as we observed in the present study, the pulmonary vasorelaxant response to isoproterenol was found to be attenuated in endothelium-intact canine pulmonary arterial rings in acute studies performed 1 h after reimplantation of the lungs (5). The cellular mechanism responsible for this effect was not investigated in that study. These results are somewhat at variance with a previous in vitro study in dogs 8 days after single lung transplantation (20). In contrast to our results, the vasorelaxant response to isoproterenol was potentiated after single lung autotransplantation in both endothelium-intact and endothelium-denuded pulmonary arterial rings (20). These differential results can possibly be ascribed to a longer pulmonary artery cross-clamp time (65 vs. 15 min), the use of a lung preservative, or timing post-lung transplantation (8 days vs. 1–14 mo) in this previous study compared with the present investigation.

In summary, we have demonstrated for the first time that activation of the \( \beta \)-adrenergic receptor signaling pathway stimulates both endothelium-dependent relaxation of pulmonary arteries by increasing the production of cGMP, as well as endothelium-independent relaxation by increasing the production of cAMP. Furthermore, LLA decreases \( \beta \)-adrenergic relaxation by selectively targeting the endothelial component of the response, and this inhibitory effect is mediated by inactivation of nitric oxide by endothelium-derived superoxide anion.

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