Effect of 5-lipoxygenase on the development of pulmonary hypertension in rats

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Effect of 5-lipoxygenase on the development of pulmonary hypertension in rats. Am J Physiol Heart Circ Physiol 286: H1775–H1784, 2004. First published January 15, 2004; 10.1152/ajpheart.00281.2003.—5-Lipoxygenase (5-LO) and its downstream leukotriene products have been implicated in the development of pulmonary hypertension. In this study, we examined the effects of 5-LO overexpression in rat lungs on pulmonary hypertension using a recombinant adenosine expressing 5-LO (Ad5-LO). Transthoracic echocardiography and right heart catheterization data showed that 5-LO overexpression in the lung did not cause pulmonary hypertension in normal rats; however, it markedly accelerated the progression of pulmonary hypertension in rats treated with monocrotaline (MCT). An increase in pulmonary artery pressure occurred earlier in the rats treated with MCT + Ad5-LO (7–10 days) compared with those treated with control vector, MCT + adenosine expressing green fluorescent protein (AdGFP), or MCT alone (15–18 days). The ratio of the right ventricle to left ventricle plus septum was higher in the MCT + Ad5-LO group than that of the MCT + AdGFP or MCT group (0.45 ± 0.08 vs. 0.35 ± 0.03 or 0.33 ± 0.06). Lung tissue histological sections from MCT + Ad5-LO rats exhibited more severe inflammatory cell infiltration and pulmonary vascular muscularization than those from MCT + AdGFP- or MCT-treated rats. Administration of 5-LO inhibitors, zileuton or MK-886, to either MCT- or MCT + Ad5-LO-treated rats prevented the development of pulmonary hypertension. These data suggest that 5-LO plays a critical role in the progression of pulmonary hypertension in rats and that the detrimental effect of 5-LO is manifest only in the setting of pulmonary vascular endothelial cell dysfunction.

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pulmonary hypertension and whether specific 5-LO inhibitors prevent the disease process.

**Methods**

*Animals.* All animals received humane care, and the procedures were approved by the Institutional Animal Care and Use Committee of Boston University. Male Sprague-Dawley rats weighing 225–250 g were obtained from Hilltop Lab Animals (Scottsdale, PA). Rats were acclimated for 4 days before initial treatment.

**Delivery of adenovirus.** Replication-deficient adenovirus expressing human 5-LO (Ad5-LO) was prepared as previously described (43). Adenovirus expressing green fluorescence protein (AdGFP) was a kind gift from Dr. John Gray at the Harvard Gene Therapy Initiative. To administer the recombinant adenovirus to rat lungs, rats were anesthetized with ketamine (100 mg/kg) and xylazine (7.5 mg/kg). Respiratory tubing was fitted to the snout of the rat and connected to the aerosol chamber of a MISTY nebulizer (Alliance Health McGaw Park, IL) containing 2 × 10^9 plaque-forming units of Ad5-LO or AdGFP in 1 ml saline. The chamber was also connected to a room air source at a flow rate of 8 l/min, causing aerosolization of the virus, and to a tank of 21% oxygen with balanced nitrogen at a flow rate of 1.5 l/min to deliver the adenovirus. The aerosolized delivery lasted 20 min for each rat.

**RNA isolation and Northern blotting.** Lung tissues were gently perfused with 40 ml of saline through the pulmonary artery, and ~300 mg of lung tissue were homogenized immediately in 10 ml TRIzol reagent (Invitrogen) followed by chloroform extraction for total RNA. Northern blotting was carried out by separating 20 µg RNA in 1.2% agarose gels containing 4% formaldehyde. The RNA was transferred onto nitrocellulose membranes and hybridized with an [α-32P]dCTP-labeled 5-LO probe, which encompasses nucleotides 45–290 of human 5-LO cDNA (GenBank Accession No. J03600). Hybridization was carried out at 68°C overnight in MiracleHyb solution (Stratagene) containing 10 µg/ml sheared salmon sperm DNA. The membrane was washed with 2 × SSC-0.1% SDS at room temperature for 15 min and then in 0.1 × SSC-0.1% SDS at 68°C for 20 min before being exposed to X-ray film. The membrane was then washed in 0.01 × SSC-0.1% SDS at 100°C twice for 15 min to strip the bound 5-LO probe and hybridized with a [α-32P]dCTP-labeled mouse GAPDH probe (Ambion), which cross-reacts with rat GAPDH, to estimate total RNA loading.

**Echocardiography.** Transthoracic echocardiography was performed on rats according to a previously described method (18). Rats were anesthetized with a ketamine-xylazine mixture as described above, and their chests were shaved before examination. Two-dimensional and Doppler imaging were performed using an Acuson C256 ultrasonographic system. Pulmonary flow acceleration time (PAAT), pulmonary flow waveform, right ventricular (RV) free wall thickness, and tricuspid regurgitation were examined in all rats on days 3, 7, 10, 15, 18, and 24.

**Hemodynamic measurements.** Rats underwent right heart catheterization at various time points during the study. Before catheterization, rats were anesthetized with the same ketamine-xylazine mixture as described above. After dissection to expose the right jugular vein, a 1.4-Fr Millar Mikro-Tip pressure catheter (Millar Instruments) was inserted into the vein and advanced to the RV. The catheter was connected to a transducer unit (Millar Instruments) and interfaced with a signal amplifier and recorder (Gould Instrument Systems), and the RV systolic pressure (RVSP) was recorded. On day 24, the systemic blood pressure of rats from each group was also measured. The left carotid artery was exposed and cannulated with a 1.4-Fr Millar pressure catheter.

**Histology.** Rat lungs were inflated with 10% phosphate-buffered formalin at a pressure of 20 cmH2O and fixed for 20 h at 4°C. The lung tissue was processed, paraffin embedded using the Hypercenter XP System and Embedding Center (Shandon; Pittsburgh, PA), and cut into 5-µm sections. The tissue sections were then heat dried on slides at 56°C for 1 h before being deparaffinized and rehydrated.

For hematoxylin and eosin staining, tissue sections were incubated in Gill-2 hematoxylin (Thermo Shandon) for 2 min, rinsed with water for 2 min, dipped once in acid alcohol (70% ethanol and 1% concentrated HCl), rinsed with water for 1 min, dipped in 1% NH4OH for 15 s, rinsed with water for 1 min, and dipped twice in 1% Eosin Y (Fisher). The stained sections were dehydrated by incubation in 95% ethanol for 2 × 30 s, 100% ethanol for 2 × 30 s, and xylene for 2 × 2.5 min and mounted with Cytoseal60 (Richard-Allan Scientific).

For trichrome staining, paraffin-embedded tissue sections were deparaffinized, rehydrated, and stained with a Masson’s Trichrome Staining Kit (Poly Scientific R&D) according to the manufacturer’s instruction. For immunohistochemistry, paraffin-embedded lung tissues were cut into 5-µm sections, heat dried on slides, deparaffinized, and rehydrated as described above. Antigen retrieval was carried out in 10 mM citric acid (pH 6) by heating for 4 × 2 min at 400 W in a microwave oven and cooling at room temperature for 20 min. The sections were washed and deionized water twice for 5 min and with PBS for 5 min before being blocked with 10% goat serum in PBS at room temperature for 30 min.

For α-actin staining, the sections were incubated with a mouse monoclonal antibody against smooth muscle cell α-actin (Sigma, 1:800 dilution in 1% immunohistochemical grade BSA-PBS) at 4°C overnight. The sections were washed in PBS twice for 5 min and incubated with the secondary antibody, biotinylated goat anti-mouse IgG (Jackson Immunoresearch), at a dilution of 1:500 in 1% BSA-PBS for 30 min at room temperature. The sections were washed with PBS twice for 5 min and incubated with complexes of avidin DH and biotinylated-alkaline phosphatase H (provided in the Vectastain ABC-AP kit, Vector Lab; Burlingame, CA) for 30 min. The sections were washed with PBS twice for 5 min and incubated with alkaline phosphatase substrate (provided in the Vector Red Substrate kit, Vector Lab) for 20 min. The slides were washed under running tap water for 5 min before being counterstained with hematoxylin, which was carried out by dipping the slides in Harris modified hematoxylin for 10 s, washing with water, dipping in acid alcohol, and rinsing with water.

For monocyte/macrophage staining, the sections were incubated with a mouse monoclonal antibody against calprotectin (clone MCA387, Chemicon; Temecula, CA, 1:50 dilution in 1% immunohistochemical grade BSA-PBS) at 4°C overnight. Calprotectin is expressed in monocytes, macrophages, and granulocytes. The sections were washed and stained with the secondary antibody as described above. The sections were developed by washing with PBS twice for 5 min, incubated with avidin and biotinylated horseradish peroxidase complex (Vectastain ABC kit) for 30 min, washed with PBS twice again for 5 min, and incubated with peroxidase substrate (DAB substrate kit, Vector Lab) for 7 min. The sections were washed with tap water and counterstained with methyl green for 4 min at 60°C, followed by rinsing in water for 1 min and in acetone containing 0.05% acetic acid for 6 min. The stained sections were dehydrated, and the slides were mounted as described above.

For 5-LO staining, the sections were incubated with rabbit anti-human 5-LO antiserum (1551 b) at 1:1,600 dilution or prebleed serum at 1:1,600 dilution (43) at 4°C overnight and developed with the same procedure as described above for α-actin staining.
Statistical analysis. Data are presented as means ± SD. Statistical analysis was performed by two-way ANOVA, with the Tukey test used for post hoc analysis. \( P < 0.05 \) indicates statistical significance.

RESULTS

Overexpression of 5-LO in rat lungs. To examine the role of 5-LO in the development of pulmonary hypertension, recombinant human 5-LO was expressed in rat lungs via adenovirus-mediated gene transfer. Approximately \( 2 \times 10^9 \) plaque-forming units of Ad5-LO were aerosolized and delivered to the rat lung through inhalation. The time course of the transgene expression in the lungs was analyzed by Northern blotting. Three groups of rats were examined: rats given vehicle (PBS), MCT, or MCT + Ad5-LO. As shown in Fig. 1, rats in the last group expressed human 5-LO mRNA; the expression peaked between 7 and 10 days and decreased by day 15 after Ad5-LO delivery.

The localization of the expressed 5-LO protein in rat lungs was examined by immunohistochemical staining. As shown in Fig. 2, the expression occurred in a wide range of cell types, including alveolar pneumocytes, bronchial epithelial cells, alveolar macrophages, and small pulmonary vessels. The antihuman 5-LO antiserum used for the study cross-reacts with rat 5-LO, which is normally expressed in leukocytes and macrophages.

Effect of 5-LO overexpression on the development of pulmonary hypertension. To determine the effect of 5-LO on the development of pulmonary hypertension in rats, five groups of rats were studied: rats given PBS, Ad5-LO, MCT, MCT + AdGFP, and MCT + Ad5-LO. All the treatments were performed on day 0.

On days 3, 7, 10, 15, 18, and 24, rats were examined by echocardiography to assess PAAT, pulmonary flow waveform, and RV free wall thickness. As shown in Fig. 3, the decrease in PAAT occurred significantly earlier in the rats treated with MCT + Ad5-LO than those treated with MCT alone (7–10 days vs. 15–18 days). Administration of MCT + AdGFP had the same effect as that of MCT alone. Rats that received Ad5-LO alone did not have decreased PAAT over the entire study period, which was similar to that observed in rats given a PBS injection. Pulmonary flow waveform change (development of a midsystolic notch) also occurred earlier in the rats treated with MCT + Ad5-LO than those treated with MCT only or MCT + AdGFP (data not shown).

![Fig. 1. Northern blotting analysis of the time course of 5-lipoxygenase (5-LO) transgene expression. Rats were treated with Dulbecco’s phosphate-buffered saline (PBS), monocrotaline (MCT), or MCT + adenovirus expressing 5-LO (Ad5-LO), and total RNA was isolated from the lung tissues of the rats on days 3, 7, 10, and 15. Northern blotting analysis was carried out using an \([\alpha-32P]CTP\)-labeled human 5-LO cDNA probe (top) and a probe against rat GAPDH (bottom). RNA samples from three individual rats were combined and examined at each time point.](http://ajpheart.physiology.org/)

![Fig. 2. Distribution of 5-LO transgene protein in rat lungs delivered by intratracheal aerosolization. Rats were treated with PBS (A) or MCT + Ad5-LO (B and C), and the lung tissues were collected on day 10 after treatment. Paraffin-embedded lung sections were stained with rabbit anti-human 5-LO antiserum (1:1,600 dilution; A and C) and rabbit prebleed serum (1:1,600 dilution; B) and developed with biotinylated-alkaline phosphatase and Vector Red substrate. The slides were counterstained with hematoxylin.](http://ajpheart.physiology.org/)
RVSPs, measured by right heart catheterization, are shown in Fig. 4 for all groups of rats on days 0, 10, 15, 18, and 24. Consistent with the echocardiographic findings, rats treated with MCT + Ad5-LO had a significantly higher RVSP on day 10 compared with that of rats treated with MCT. Rats treated with MCT + AdGFP behaved similarly to rats treated with MCT alone. Administration of Ad5-LO alone or PBS did not cause increases in RVSP. By day 24, no significant difference in RVSP was found among the MCT + Ad5-LO, MCT + AdGFP, and MCT-alone treatment groups.

Changes in the RV-to-left ventricle plus septum weight ratio. Rats in the above treatment groups were killed at day 26. Their systolic pressures and body weights were measured, and hearts were dissected. The weight ratio of the RV to left ventricle plus septum (RV/LV+S) was calculated. As shown in Fig. 5, the ratio was significantly higher in the group treated with MCT + Ad5-LO than in the groups treated with MCT + AdGFP or MCT alone (0.45 ± 0.08 vs. 0.35 ± 0.03 or 0.33 ± 0.06, P < 0.02). In addition, the ratios in the groups treated with MCT were significantly higher than those without MCT. Because the MCT + Ad5-LO-treated group were exposed to a significantly longer period of increased pulmonary artery pressure, it may explain the higher RV/LV+S found in this group, even though its RVSP was no longer higher than that of the MCT and MCT + AdGFP treatment groups by day 24. There was no significant difference in systolic blood pressure or heart rate among the five groups at day 26 (data not shown).

Histological changes. Lungs from the treated rats were harvested at various time points and were fixed in formalin. After paraffin embedding and sectioning, the tissues were stained with hematoxylin and eosin. Three major histological changes were found in the MCT-treated groups: inflammatory cell infiltration, muscularization of the distal pulmonary vessels, and fibrosis of the interstitium. These changes in the MCT + Ad5-LO group were more severe than those in the MCT + AdGFP or MCT-alone groups. The sections also show scattered postinflammatory extension of bronchiolar epithelium to thickened septa, which appeared to be similar in all MCT-treated groups (data not shown). To compare the degree...
of inflammation and muscularization of distal vessels, lung tissue sections were stained with specific antibodies against monocytes/macrophages or smooth muscle cell α-actin, and the numbers of the antibody-stained cells or vessels were counted. In addition, Masson’s trichrome staining was performed on tissue sections collected at day 26 after treatment to monitor the fibrosis in these sections.

Figure 6A shows the photomicrographs of hematoxylin and eosin-stained lung sections prepared from rats treated with PBS, MCT, MCT + AdGFP, and MCT + Ad5-LO at day 15. The staining revealed the presence of inflammatory cells, predominantly monocytes/macrophages (include enlarged foam cells) and lymphocytes. The distribution of the infiltrated inflammatory cells in the lung is patchy, many being in the proximity of blood vessels. To estimate the degree of inflammation, lung sections from MCT, MCT + AdGFP, and MCT + Ad5-LO treatment groups collected at days 7, 15, and 22 were stained with anti-monocyte/macrophage antibody, and the number of stained cells was counted. As shown in Fig. 6B, the number of macrophages in the MCT + AdGFP and

![Image of photomicrographs and bar graph showing inflammatory cell infiltration.]
MCT + Ad5-LO groups was the highest at day 15 and was reduced by day 22. The number of macrophages in the MCT group was increased by day 22. The MCT + Ad5-LO group had significantly more macrophage infiltration than the MCT + AdGFP group at day 15. The MCT-only group had noticeably less macrophage infiltration than the MCT + Ad-LO and MCT + AdGFP groups, indicating that part of the inflammation was caused by adenosine infection.

Shown in Fig. 7A are photomicrographs of an anti-smooth muscle cell α-actin-stained lung sections prepared from rats treated with PBS, MCT + AdGFP, and MCT + Ad-LO at day 22. The muscularization and thickening of distal pulmonary vessels was more visible in the MCT + Ad-LO group than the MCT + AdGFP group. The numbers of the muscularized distal vessels were counted in groups treated with MCT + Ad-LO and MCT + AdGFP at days 7, 15, 22, and 26. As shown in Fig. 7B, the number of muscularized distal pulmonary vessels increased significantly at day 15 and reached a plateau by day 22. The MCT + Ad-LO treatment group displayed a trend toward higher numbers of muscularized vessels than those of the MCT + AdGFP treatment group on days 22 and 26, but the difference did not reach statistical significance.

Figure 8 shows representative photomicrographs of lung sections stained with Masson’s trichrome stain from rats treated with PBS, MCT + AdGFP, or MCT + Ad-LO at day 26. As demonstrated, the lung sections had extensive collagen deposition, consistent with fibrosis. The MCT + Ad-LO treatment group appeared to have more alveolar thickening and collagen deposition than the MCT + AdGFP treatment group.

Effect of 5-LO inhibition on the development of pulmonary hypertension. To examine whether these effects of 5-LO are dependent on its activity, two specific inhibitors, one for 5-LO (zileuton) and one for FLAP (MK-886), were used. Rats were treated with MCT + Ad-LO on day 0, and DMSO (solvent for MK-886), MK-886, or zileuton was administered on day 1 and daily thereafter. The 1-day delay of the administration of inhibitors was designed to avoid potential interference of the activation of MCT in the liver. Right heart catheterization was performed on all groups on days 10, 15; and 26. As shown in Fig. 9A, both inhibitors prevented the increase of RV pressure in the MCT + Ad5-LO-treated rats at day 10 (15 ± 2 and 16 ± 4 mmHg for MCT + Ad5-LO + MK-886 and MCT + Ad-5-LO + zileuton, respectively, vs. 25 ± 2 mmHg for MCT + Ad5-LO, P < 0.05). Interestingly, MK-886 and zileuton also prevented the late development of pulmonary hypertension in the rats (day 26: MCT + Ad5-LO = 42 ± 4 mmHg vs. 25 ± 5 and 22 ± 8 mmHg for MCT + Ad5-LO + MK-886 and MCT + Ad5-LO + zileuton rats, respectively, P < 0.05 vs. MCT + Ad5-LO).

To examine the possibility that 5-LO plays a role in MCT-induced pulmonary hypertension in rats, rats were treated with MCT only and received daily administration of vehicle (DMSO), MK-886, or zileuton as described above. Right heart catheterization was performed on all groups on day 35. As shown in Fig. 9B, both MK-886 and zileuton prevented the development of pulmonary hypertension in the MCT-treated rats, which suggests that endogenous 5-LO played an important role in mediating MCT-induced pulmonary hypertension in rats.

DISCUSSION

This study examined the role of 5-LO in the development of pulmonary hypertension by overexpressing the enzyme in rat lungs through adenovirus-mediated gene transfer and by administering specific 5-LO inhibitors to MCT-treated rats. The results show that overexpression of 5-LO in normal lungs did not cause pulmonary hypertension; however, it markedly accelerated the development of pulmonary hypertension in rats treated with MCT. 5-LO inhibitors inhibited the effect of 5-LO and also prevented MCT-induced pulmonary hypertension.

Two questions arise from the results of this study: 1) Why did the overexpression of 5-LO have an adverse effect on the pulmonary vasculature in MCT-treated rats but not in normal rats? and 2) Why did the 5-LO inhibitors prevent the pulmonary hypertension caused by MCT?

The first question can be explained by two possibilities. One is that the normal pulmonary vasculature is somewhat protected from leukotriene-dependent vasoconstriction, but in the MCT-injured pulmonary vessels, this protection is weakened. The source of the protection has been implicated by Bäck and colleagues (4), who reported that cysteinyl leukotrienes stimulate prostacyclin production in the isolated human pulmonary artery and that the endothelium-denuded or endomethacin-treated human pulmonary artery is significantly more sensitive to cysteinyl leukotriene-induced vasoconstriction (in an organ chamber) than endothelialized pulmonary arteries. The second possibility is that MCT-treated rats had more pulmonary inflammation than normal rats, and the inflammation provided a supportive environment for 5-LO-catalyzed leukotriene biosynthesis. Leukotriene biosynthesis consists of three major steps: release of arachidonic acid from cell membranes by phospholipase A2, conversion of arachidonic acid to leukotriene A4 by 5-LO, and transformation of leukotriene A4 to leukotrienes B4 or cysteinyl leukotrienes. Both phospholipase A2 and 5-LO require calcium for their activity, and intracellular calcium release is related to the state of cell activation. Inflammatory stimuli can cause activation of a variety of cells. Further analysis of prostacyclin and leukotriene release in the normal and dysfunctional pulmonary vasculature in animals expressing 5-LO in the lung would help to confirm these hypotheses.

The finding that 5-LO inhibitors prevented the MCT-induced pulmonary hypertension suggests endogenous 5-LO is essential for MCT-induced pulmonary hypertension. MCT-induced pulmonary endothelial injury is known to be followed by significant pulmonary vascular and alveolar inflammation (34, 40), and the latter is thought to be important in subsequent pulmonary vascular remodeling and pulmonary hypertension. Previous studies have shown that inhibition of inflammation by interleukin-1 receptor antagonists (37) or by an antibody that neutralizes monocyte chemoattractant and activating factor monocyt e chemoattractant protein-1 (19) alleviate MCT-induced pulmonary hypertension. As 5-LO is predominantly expressed in inflammatory cells, infiltration of these cells in the lung results in an increase in 5-LO in the tissue. Downstream leukotriene products of 5-LO could markedly amplify the inflammatory process by attracting more inflammatory cells to the lung (leukotriene B4) and by increasing vascular permeability to facilitate cell infiltration. It is possible that 5-LO inhibitors prevented the MCT-induced pulmonary hyperten-
sion via a similar mechanism as that by other inflammatory inhibitors.

In addition to the facilitation of inflammatory processes, 5-LO could exert its effect through several other possible mechanisms. These include 1) causing pulmonary vasoconstriction by increasing cysteinyl leukotriene production; 2) promoting vascular cell proliferation by its nuclear localization or through interaction with cytoskeletal proteins (21, 27, 29);
and 3) altering TGF-β superfamily signaling through binding to TRAP-1. To address these mechanisms, further studies need to explore 1) the types of cysteinyl leukotriene receptors on pulmonary arteries or arterioles and the potential influence of endothelial dysfunction on the function of these receptors; 2) the role of the cytoskeleton, actin, or tyrosine kinase signaling in vascular remodeling during the development of pulmonary arterial hypertension; and 3) the effect of the 5-LO-TRAP-1 interaction on TGF-β signaling. This latter interaction may be of particular relevance to familial and sporadic forms of primary pulmonary hypertension in which imbalanced TGF-β superfamily signaling is believed to play a role in the disease development (20, 35, 36, 42). Because 5-LO overexpression has been demonstrated in primary pulmonary hypertension, examining these possibilities in the future would help clarify the specific role of 5-LO in pulmonary hypertension as well as the mechanism of the development of pulmonary hypertension in general.

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