Attenuation of Hypoxic Pulmonary Vasoconstriction by Endotoxemia Requires 5-Lipoxygenase in Mice

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Abstract—Sepsis and endotoxemia impair hypoxic pulmonary vasoconstriction (HPV), thereby reducing systemic oxygenation. To assess the role of leukotrienes (LTs) in the attenuation of HPV during endotoxemia, the increase in left lung pulmonary vascular resistance (LPVR) before and during left mainstem bronchus occlusion (LMBO) was measured in mice with and without a deletion of the gene encoding 5-lipoxygenase (5-LO). LMBO increased the LPVR equally in saline-challenged wild-type and 5-LO–deficient mice (96±20% and 94±19%, respectively). Twenty-two hours after challenge with Escherichia coli endotoxin, the ability of LMBO to increase LPVR was markedly impaired in wild-type mice (27±7%; P<0.05) but not in 5-LO–deficient mice (72±9%) or in wild-type mice pretreated with MK886, an inhibitor of 5-LO activity (76±10%). Compared with wild-type mice, endotoxin-induced disruption of lung structures and inflammatory cell influx in the lung were markedly attenuated in 5-LO–deficient mice. Administration of MK571, a selective cysteinyl LT, receptor antagonist, 1 hour before endotoxin challenge preserved HPV and attenuated pulmonary injury in wild-type mice but did not prevent the endotoxin-induced increase in pulmonary myeloperoxidase activity. Taken together, these findings demonstrate that a 5-LO product, most likely a cysteinyl LT, contributes to the attenuation of HPV and to pulmonary injury after challenge with endotoxin. (Circ Res. 2001;88:832-838.)

Key Words: cysteinyl leukotrienes ■ pulmonary injury ■ left mainstem bronchus occlusion

H ypoxic pulmonary vasoconstriction (HPV) is characterized by vasoconstriction of pulmonary vessels in poorly ventilated hypoxic lung regions, thus optimizing pulmonary gas exchange. The sensor and effector mechanisms responsible for HPV reside in vascular smooth muscle cells of pulmonary arterioles1,2; however, the precise mechanisms responsible for the sepsis-induced attenuation of HPV remain incompletely understood.3

HPV is markedly impaired in patients with clinical sepsis or the acute respiratory distress syndrome (ARDS).4,5 Experimental endotoxemia has also been shown to impair HPV in several animal species.6,7 Although the mechanisms responsible for the sepsis-induced attenuation of HPV remain incompletely elucidated, various inflammatory mediators including prostaglandins,8 thromboxanes,9 platelet-activating factor,6 and cytokines10 have all been implicated. Recently, we reported that increased pulmonary NO levels are necessary, but not sufficient, to impair HPV in a murine sepsis model.11 These studies suggested that, in addition to increased pulmonary NO levels, the attenuation of HPV after endotoxin challenge requires unknown endotoxin-induced inflammatory products.

Leukotrienes (LTs) are potent lipid mediators of inflammation derived from arachidonic acid (AA) metabolism.12 Synthesis of LTs is initiated by the conversion of AA to HPETE by arachidonate 5-lipoxygenase (5-LO) in the presence of 5-LO–activating protein (FLAP). This intermediate can be dehydrated to the epoxide intermediate LTAA, which can either be hydrolyzed to form LTB4 by LTA4 hydrolase or conjugated with glutathione by LTC4 synthase to form the cysteinyl LTs (cysLTs; LTC4, LTD4, and LTE4). Whereas the role of LTB4 as a potent chemokinetic and chemotactic agent for the polymorphonuclear neutrophil (PMN) is well established, cysLTs appear to have multiple actions including bronchoconstriction, modulation of vascular smooth muscle tone, proliferation of smooth muscle, edema formation, and stimulation of eosinophil migration.13,14 The actions of LTB4 are largely mediated via 2 G protein–coupled LTB4 receptors (BLT1 and BLT2),15,16 whereas the effects of cysLTs are mediated via cysLT1 and cysLT2.17,18

LTs have also been implicated as possible mediators of endotoxin-induced acute lung injury. The presence of LTs in the bronchoalveolar lavage (BAL) fluid of patients with ARDS, as well as increased cysLT levels in lung tissue of endotoxin-challenged rodents, has been reported.19,20 Infusion of LTs into animals produces acute lung injury resembling the clinical presentation of endotoxemia and ARDS, includ-
deficient24 and LTA4 hydrolase–deficient mice,25 as well as their respective wild-type littermates, were maintained at the Massachusetts General Hospital Animal Resource Facility.

Measurement of Hypoxic Pulmonary Vasoconstriction in Mice

Surgical preparation of mice for hemodynamic study after thoracotomy was performed as described previously.11,20 Systemic arterial pressure (SAP) and pulmonary artery pressure (PAP) were continuously monitored using biomedical amplifiers (Hewlett Packard, model 8805C). Left pulmonary artery blood flow (QLPA) was measured with a small-vessel flow probe connected to a flowmeter (T106; Transonic Instruments). All measured signals were digitally recorded using a data acquisition system (DI 720; Dataq Instruments).

To induce regional (left lung) alveolar hypoxia, the left mainstem bronchus was reversibly occluded with a microvascular clip. Complete collapse of the left lung was visually observed within about a minute and confirmed by transient overinflation of the right lung. PAP, SAP, and QLPA were continuously measured during left mainstem bronchus occlusion (LMBO).

Detailed methods for measurements of left lung pulmonary vascular resistance (LPVR) and description of experimental groups are available in the online data supplement available at http://www.circresaha.org.

RNA Blot Hybridization

RNA was extracted from lungs using the guanidine isothiocyanate–cesium chloride method. RNA (15 μg) was fractionated in formaldehyde-agarose gels containing ethidium bromide, photographed, and transferred to nylon membranes. Membranes were hybridized with a 32P-labeled 0.3-kb mouse inducible NO synthase (NOS2) cDNA probe.27 Autoradiograms and photographs were scanned using a Color Image Scanner (Seiko Epson Corp) and the NIH Image 1.44 software. To estimate pulmonary NOS2 mRNA concentrations, the NOS2 mRNA:28S ribosomal RNA ratio was determined by dividing the absorbance corresponding to the NOS2 cDNA probe hybridization on autoradiographs by the absorbance corresponding to the 28S ribosomal RNA on photographs.

Materials and Methods

All animal experiments were conducted under protocols reviewed and approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital. The 5-LO–deficient mice (B6;129S-AloxsflK/J and their wild-type controls (B6129S2F2J)) were obtained from the Jackson Laboratory (Bar Harbor, ME). BLT1-deficient27 and LTA4 hydrolase–deficient mice,25 as well as their respective wild-type littermates, were maintained at the Massachusetts General Hospital Animal Resource Facility.

Reagents

MK886, a FLAP inhibitor, was purchased from Calbiochem. MK571, a cysLT, receptor antagonist, was purchased from Cayman Chemical Company. Escherichia coli 0111:B4 endotoxin was purchased from Difco Laboratories. NO gas was purchased from INO Therapeutics. All other chemicals were purchased from Sigma.

Results

Effects of Unilateral Alveolar Hypoxia on Pulmonary Blood Flow

HPV was assessed as the change in the slope of the left lung pulmonary flow–pressure relationship before and 5 minutes after occlusion of the LMBO. LMBO after saline challenge markedly decreased QLPA without changing PAP or SAP in all mice (Figure 1A, see online Table 1 in the data supplement available at http://www.circresaha.org). The PAP-QLPA relationship was analyzed from the plot generated by reducing cardiac output with transient inferior vena cava occlusion before and after LMBO, in which incremental LPVR was represented by the slope (Figure 1B). The increase in LPVR
in response to LMBO was similar in saline-challenged wild-type mice (96±20%) and in saline-challenged 5-LO–deficient mice (94±19%) (Figure 2A). These results imply that 5-LO and its products are not necessary to produce HPV in healthy mice.

**Effect of Endotoxemia on Pulmonary and Systemic Hemodynamics During Unilateral Alveolar Hypoxia**

Before LMBO, hemodynamic parameters did not differ between saline-challenged mice and endotoxin-challenged mice (see online Table 1). Five minutes after LMBO, the LPVR increased by only 27±7% in endotoxin-challenged wild-type mice (versus 96±20% in saline-challenged wild-type mice; *P*<0.05; Figures 1C, 1D, and 2A). In contrast, the LMBO-induced increase of LPVR was 76±9% in endotoxin-challenged 5-LO–deficient mice (Figure 2A) and did not differ from the LMBO-induced increase in LPVR in saline-challenged mice of either strain, demonstrating that HPV is preserved in endotoxin-challenged 5-LO–deficient mice.

Because potential strain differences in the background of wild-type mice and 5-LO–deficient mice might lead to a differing response to endotoxin,29 we sought to confirm that 5-LO deficiency preserved HPV after endotoxin challenge by studying wild-type mice pretreated with the FLAP inhibitor MK886. The increase in the LPVR in response to LMBO was greater in endotoxin-challenged wild-type mice pretreated with MK886 than in endotoxin-challenged wild-type mice (Figure 2A). These results suggest that decreased 5-LO activity protects mice from endotoxin-induced HPV attenuation by preventing NOS2 induction. To test this hypothesis, pulmonary NOS2 mRNA levels were measured 7 hours after intraperitoneal administration of saline or endotoxin in wild-type and 5-LO–deficient mice. This time point was chosen because pulmonary NOS2 mRNA levels were maximal 6 to 8 hours after wild-type mice were challenged with endotoxin (data not shown). NOS2 mRNA levels were not detectable in lungs of mice of either genotype 7 hours after saline challenge. In contrast, 7 hours after endotoxin challenge, NOS2 gene expression was induced in lungs of wild-type and 5-LO–deficient mice. The NOS2 mRNA:28S ribosomal RNA ratio did not differ significantly in lungs from wild-type and 5-LO–deficient mice. This time point was chosen because pulmonary NOS2 mRNA levels were maximal 6 to 8 hours after wild-type mice were challenged with endotoxin (data not shown). NOS2 mRNA levels were not detectable in lungs of mice of either genotype 7 hours after saline challenge. In contrast, 7 hours after endotoxin challenge, NOS2 gene expression was induced in lungs of wild-type and 5-LO–deficient mice. The NOS2 mRNA:28S ribosomal RNA ratio did not differ significantly in lungs from wild-type and 5-LO–deficient mice (1.3±0.2 and 1.0±0.1, respectively; *n*=5 in each group; *P*=0.1; see online Figure 1 in the data supplement available at http://www.circresaha.org).

**Prolonged Inhalation of 40 ppm NO**

To confirm that decreased endotoxin-induced pulmonary NO concentrations did not account for the protective effects of 5-LO deficiency on HPV, endotoxin-challenged 5-LO–deficient mice breathed 40 ppm NO in air for 22 hours to potentially replace molecular NO in the lung during endotoxemia. The hemodynamic studies were performed 1 hour after NO inhalation was discontinued, allowing ample time for any potential vasodilator action of inhaled NO to dissipate. Breathing 40 ppm NO for 22 hours after endotoxin challenge did not impair HPV in 5-LO–deficient mice (*n*=4); the increase in the LPVR after LMBO was 80±24% in endotoxin-challenged 5-LO–deficient mice after breathing 40 ppm NO for 22 hours.

**Pulmonary Injury Results From Endotoxemia**

**Lung Wet/Dry Weight Ratios**

Endotoxin challenge increased lung wet/dry weight ratios equally in wild-type mice (wet weight/dry weight, 5.0±0.2, *P*<0.05 versus saline-challenged wild-type mice, 4.2±0.2)
and in 5-LO–deficient mice (5.0±0.1, *P<0.05 versus saline-challenged 5-LO–deficient mice, 4.3±0.1).

**MPO Activity**

Lung MPO activity was >10-fold higher in endotoxin-challenged wild-type mice than in saline-challenged wild-type mice (*P<0.01, Figure 3). Lung MPO activity in endotoxin-challenged 5-LO–deficient mice was greater than in saline-challenged 5-LO–deficient mice (*P<0.01, Figure 3). However, pretreatment with MK886 attenuated the increase in lung MPO activity in endotoxin-challenged wild-type mice (Figure 3), confirming the reduction of lung MPO activity in the presence of decreased 5-LO activity.

**Lung Morphology**

To learn whether endotoxin-induced attenuation of HPV and its modulation by 5-LO deficiency was associated with structural alterations of the lung, lung sections from endotoxin-challenged wild-type mice and endotoxin-challenged 5-LO–deficient mice were examined microscopically. Endotoxin challenge disrupted the normal alveolocapillary membrane structure and increased the number of inflammatory cells (ie, PMNs and monocytes), predominantly within the intravascular space of wild-type mice (Figure 4B). Alveolar-capillary membrane changes were diffusely present throughout the lung and included interstitial edema and epithelial type I cell swelling. Compared with the endotoxin-challenged wild-type mouse, in the endotoxin-challenged 5-LO–deficient mouse (Figure 4D), membrane changes were less marked, and fewer inflammatory cells were seen as focal collections within the intravascular space.

**Circulating Leukocytes**

In wild-type mice, endotoxin challenge markedly decreased the number of circulating leukocytes (control, 4.9±0.4×10^3/μL; endotoxin challenged, 2.2±0.4×10^3/μL; *P<0.01 versus control). In contrast, endotoxin challenge did not alter the circulating leukocyte count in 5-LO–deficient mice (control, 5.1±0.2×10^3/μL; endotoxin challenged, 4.8±0.3×10^3/μL).

**LT Levels in BAL Fluids**

To learn whether alveolar LT levels are increased after endotoxin challenge in this model, the concentrations of cysLTs and LTβ were measured in BAL fluid 22 hours after wild-type or 5-LO–deficient mice were challenged with endotoxin or saline. LTβ and cysLT levels in BAL fluid from saline-challenged wild-type mice were 41±11 and 27±8 pg/mL, respectively. Levels of LTβ and cysLTs in BAL fluid were increased 22 hours after endotoxin challenge in wild-type mice (58±15 and 65±18 pg/mL, respectively; *P<0.05 for both versus saline-challenged wild-type mice). LTs were not detectable in BAL fluid from 5-LO–deficient mice either at baseline or 22 hours after endotoxin challenge. These results suggest that increased LT levels are associated with endotoxin-induced attenuation of HPV in mice.
Endotoxin-Induced Attenuation of HPV

Because LTB4 levels were increased in BAL fluid from endotoxin-challenged wild-type mice, effects of the selective cysLT1 receptor antagonist, MK571, on the endotoxin-induced attenuation of HPV were examined in wild-type mice; the increase in the LPVR in response to LMBO was greater in endotoxin-challenged wild-type mice pretreated with MK571 than in endotoxin-challenged wild-type mice pretreated with saline (P<0.01; Figure 2A). The LMBO-induced increase in the LPVR in saline-challenged wild-type mice pretreated with MK571 did not differ from that in mice challenged with saline alone (data not shown). These results suggest that cysLTs play an important role in the attenuation of HPV 22 hours after endotoxin challenge of wild-type mice.

To determine whether acute pharmacological blockade of the cysLT1 receptor 22 hours after endotoxin challenge augments HPV during LMBO, hemodynamic studies were performed in wild-type mice that had received an intravenous bolus injection of MK571 (1 mg/kg) 22 hours after challenge with saline or endotoxin. In saline-challenged wild-type mice, acute administration of MK571 (n=3) did not alter the LMBO-induced increase in the LPVR (99±13% before MK571; 96±23% after MK571). All other measured hemodynamic parameters did not change after injection of MK571. In wild-type mice challenged with endotoxin 22 hours earlier (n=4), acute MK571 administration did not augment the LPVR response to LMBO (34±4% before MK571; 24±6% after MK571). Because it would be anticipated that the plasma concentration of MK571 would be highest after acute IV administration, these results suggest that the preservation of HPV in endotoxin-challenged wild-type mice pretreated with MK571 was not attributable to acute hemodynamic effects of MK571.

To assess the impact of MK571 administration on pulmonary PMN accumulation, MPO activity was measured in lung tissue samples harvested from MK571-pretreated wild-type mice at 7 hours after IP administration of 10 mg/kg endotoxin or saline. The increase in lung MPO activity in endotoxin-challenged wild-type mice was not attenuated by pretreatment with the cysLT1 receptor antagonist MK571 (Figure 3).

Pretreatment with MK571 attenuated the thickening and focal disruption of the alveolar-capillary membrane of endotoxin-challenged wild-type mice (Figure 4C). Compared with saline-challenged wild-type mice, however, the overall cellularity and the number of inflammatory cells were increased in endotoxin-challenged wild-type mice pretreated with MK571.

Discussion

Our results suggest that in mice 5-LO plays an important role in the endotoxin-induced attenuation of HPV. A congenital deficiency of 5-LO not only protected mice from endotoxin-induced attenuation of HPV but also decreased the pulmonary inflammatory cell infiltrate and the disruption of alveolar-capillary membrane structure. Furthermore, pretreatment with MK571, a highly specific cysLT1 receptor antagonist, conferred protection against endotoxin-induced attenuation of HPV. Pretreatment with MK571 protected the lungs of...
endotoxin-challenged wild-type mice from alveolar membrane changes. These observations indicate that cysLTs appear to play a crucial role in endotoxin-induced lung injury and attenuation of HPV in mice.

The observation that the increase in LPVR in response to LMBO did not differ in saline-challenged 5-LO–deficient mice and saline-challenged wild-type mice is consistent with previous studies in other species, indicating that 5-LO and its products are not required for HPV in normal animals. We also studied LTA4 hydrolase–deficient mice, and found that endotoxin challenge also impaired HPV in these mice, and in wild-type mice pretreated with MK571. These results are consistent with previous reports describing increased LT levels in the BAL fluid obtained from endotoxin-challenged wild-type mice. These observations indicate that absences in LT levels at the time of hemodynamic measurements; intravenous bolus administration of MK571, given 15 minutes before the measurement of LPVR, did not restore HPV 22 hours after endotoxin challenge. Although we measured increased LT levels in BAL fluid 22 hours after endotoxin challenge, it is likely that increased LT levels in lung tissue were consistently present at earlier times. Lung tissue levels of LTC4 are elevated for the first 3 hours after endotoxin challenge in rats. Therefore, it is probable that increased cysLT levels were present in the lung at earlier times after endotoxin challenge and that MK571 blocked the deleterious effects of cysLTs during this period.

Exposure to endotoxin stimulates cells to express NOS2. We have recently reported that increased pulmonary NO levels (produced by NOS2 or inhaled at high levels from exogenous sources) are necessary to impair HPV in this murine sepsis model. Although NOS2-deficient mice had preserved HPV after endotoxin challenge, when NO was replaced by inhaling 40 ppm NO, NOS2-deficient mice had impaired HPV. To learn whether the protective effects of 5-LO deficiency on HPV are mediated by preventing the endotoxin-induced increase in pulmonary NO levels, we studied whether replenishing pulmonary NO levels via inhalation could impair HPV in endotoxin-challenged 5-LO–deficient mice. In contrast to endotoxin-challenged NOS2-deficient mice, HPV was preserved in endotoxin-challenged 5-LO–deficient mice breathing 40 ppm NO for 22 hours, which suggests that the protective effects of 5-LO deficiency against endotoxin-induced attenuation of HPV were not mediated by inhibiting the endotoxin-induced increase of pulmonary NO concentrations. These results were supported by the finding that the endotoxin-mediated induction of pulmonary NOS2 gene expression did not differ significantly in wild-type and 5-LO–deficient mice.

We found that the changes in alveolar-capillary membrane structure induced by endotoxin were attenuated by either 5-LO deficiency or MK571 pretreatment. These findings are consistent with the recent report that disruption of the gene encoding cytosolic phospholipase A2, an enzyme necessary for AA release from the glycosphospholipid membrane, protected mice from lipopolysaccharide/zymosan–induced lung injury. These observations suggest that cytosolic phospholipase A2–initiated pathways, such as biosynthesis of cysLTs, play an important role in the production of murine acute lung injury.

In summary, we investigated the roles of 5-LO and the LTs in endotoxin-induced attenuation of HPV. We found that a congenital deficiency of 5-LO protected mice from endotoxin-induced attenuation of HPV. Pretreatment with MK571, a cysLT1-receptor antagonist, preserved HPV in endotoxin-challenged wild-type mice, whereas acute treatment after 22 hours with MK571 did not restore HPV. Protective effects on HPV of cysLT1-receptor blockade were not associated with an attenuated increase of lung tissue MPO activity, suggesting that the mechanism by which cysLTs...
impair HPV may involve targets other than leukocytes. Although reasoning from mice to humans requires many continuing research steps, our results suggest a potential role for pretreatment with selective inhibitors of the 5-LO pathway, especially cysteine leukotriene receptor antagonists, to prevent the attenuation of HPV in patients with clinical sepsis.

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