Hypoxic 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 arterioles; however, the precise mechanisms responsible for the sepsis-induced attenuation of HPV remain incompletely understood.

HPV is markedly impaired in patients with clinical sepsis or the acute respiratory distress syndrome (ARDS). Experimental endotoxemia has also been shown to impair HPV in several animal species. Although the mechanisms responsible for the sepsis-induced attenuation of HPV remain incompletely elucidated, various inflammatory mediators including prostaglandins, thromboxanes, platelet-activating factor, and cytokines 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. 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. 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 LTA₄, 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. The actions of LTB₄ are largely mediated via 2 G protein–coupled LTB₄ receptors (BLT1 and BLT2), whereas the effects of cysLTs are mediated via cysLT₁ and cysLT₂.

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. Infusion of LTs into animals produces acute lung injury resembling the clinical presentation of endotoxemia and ARDS, includ-
ing pulmonary hypertension and increased vascular permeability resulting in pulmonary edema and hypoxemia. However, studies examining the effects of pharmacological inhibitors of 5-LO and LT receptors on endotoxin-induced lung injury have yielded conflicting results, some showing protection and others showing no protection.

The objective of our study was to learn whether LTs play an important role in endotoxin-induced attenuation of HPV. We measured pulmonary blood flow redistribution in response to unilateral hypoxia in the mouse 22 hours after endotoxin challenge. We report that 5-LO and its products, especially cysLTs, are required to impair HPV after endotoxin challenge.

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-Alox5fltm1/aj and their wild-type controls (B6129SF2/J) were obtained from the Jackson Laboratory (Bar Harbor, ME). BLT1-deficient and LTA4 hydrolase–deficient mice, 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.

Measurement of Hypoxic Pulmonary Vasoconstriction in Mice

Surgical preparation of mice for hemodynamic study after thoracotomy was performed as described previously. 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; Datasq Instraments).

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. SAP, PAP, and QLPA were continuously monitored during left mainstem bronchus occlusion (LMOB).

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. 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.

Measurement of LT Concentrations

Twenty-two hours after challenge with endotoxin or saline, BAL was performed using 5 × 1 mL PBS in additional wild-type and 5-LO–deficient mice. cysLT and LTB4 concentrations in BAL fluid supernatants were measured by using enzyme immunoassay kits (Cayman Chemical Company) as previously described.

Lung Wet/Dry Ratio

At the end of hemodynamic studies 22 hours after endotoxin challenge, mice were euthanized with pentobarbital (0.1 mg/g IP), and both lungs, excluding hilar structures, were excised, blotted, and immediately weighed. Thereafter, the tissue was dried in a micro-wave oven for 10 minutes and reweighed, as described previously. The lung wet/dry weight ratio was calculated.

Myeloperoxidase (MPO) Assay

Seven hours after intraperitoneal injection of E. coli endotoxin 10 mg/kg or saline, additional wild-type mice and 5-LO–deficient mice received a lethal injection of pentobarbital (0.1 mg/g), and their lungs were removed, rinsed with PBS, blotted dry, and weighed. PMN infiltration in lung tissue was estimated by measuring MPO activity, as previously described. MPO activity assay was performed 7 hours after endotoxin challenge on the basis of a pilot study that showed that MPO activity was greatest ~7 hours after endotoxin challenge.

Lung Morphology

Seven hours after endotoxin challenge, additional wild-type mice, with and without MK571 pretreatment, and 5-LO–deficient mice were euthanized, and their lungs were perfusion-fixed with 3% paraformaldehyde and 0.1% glutaraldehyde via both airway and pulmonary artery. Histo-resin sections (2 μm thickness) of the fixed lungs were stained with toluidine blue and microscopically examined by an investigator blinded as to the treatment and genotype of the mice.

Circulating Leukocyte Count

Using a Coulter counter (model Ac · T diff, Beckman Coulter), circulating leukocytes were counted in heparinized blood samples from additional wild-type and 5-LO–deficient mice 7 hours after endotoxin challenge.

Statistical Analysis

The LMOB-induced increase in LPVR was expressed as the percentage increase from the baseline LPVR before LMOB. Differences between groups were determined using a 2-way ANOVA with repeated measures. When significant differences were detected by ANOVA, a post hoc Newman-Keuls test was used (Statistica for Windows version 5.0, StatSoft Inc). A P value <0.05 was considered a significant difference. All data are expressed as mean ± SEM.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

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 LMOB. LMOB 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 LMOB, 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, we sought to confirm that 5-LO deficiency protected HPV against 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. The NOS2 mRNA levels did not account for the protective effects of 5-LO deficiency on HPV in 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 versus 4.2±0.1)
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) but was less than the MPO activity of lungs from endotoxin-challenged wild-type mice (P<0.05, Figure 3). Furthermore, 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 alveolar-capillary membrane structure and increased the number of inflammatory cells (ie, PMNs and monocyte subsets), 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 LTB_4 were measured in BAL fluid 22 hours after wild-type or 5-LO–deficient mice were challenged with endotoxin or saline. LTB_4 and cysLT levels in BAL fluid from saline-challenged wild-type mice were 41±11 and 27±8 pg/mL, respectively. Levels of LTB_4 and cysLTs in BAL fluid from saline-challenged wild-type mice were 41±11 and 27±8 pg/mL, respectively. Levels of LTB_4 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). LTB_4s 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.
Contributions of LTB4 and cysLTs to Endotoxin-Induced Attenuation of HPV

Because LTB4 levels were increased in BAL fluid from endotoxin-challenged wild-type mice, we examined the effects on HPV of a congenital deficiency of BLT1, a receptor for LTB4 or LTA4 hydrolase, an enzyme necessary for production of LTB4. Congenital deficiency of either BLT1 or LTA4 hydrolase did not affect the magnitude of the LMOB-induced increase in LPVR after saline challenge (Figures 2B and 2C), implying that LTB4 is not necessary for HPV in the healthy mouse. The LMOB-induced increase in the LPVR was reduced equally in endotoxin-challenged BLT1-deficient mice and endotoxin-challenged BLT1-competent wild-type littermates (Figure 2B). Similarly, after endotoxin challenge, the LMOB-induced increase in LPVR was decreased equally in LTA4 hydrolase-deficient mice and LTA4 hydrolase-competent wild-type littermates (Figure 2C). These results suggest that LTB4 does not contribute to the attenuation of HPV in endotoxin-challenged mice.

Because cysLT 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 LMOB 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 LMOB-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 LMOB, 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 LMOB-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 LMOB (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 LMOB 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 found that a congenital deficiency of 5-LO–deficient mice that had preserved HPV suggests that an increase in pulmonary LT concentrations contributes to the attenuation of HPV.

To better characterize the roles of LTβ and cysLTs in endotoxin-induced attenuation of HPV, we measured the increase of LPVR in response to LMOB after endotoxin challenge in BLT1-deficient mice, in LTA4 hydrolase–deficient mice, and in wild-type mice pretreated with MK571. We found that a congenital deficiency of BLT1 did not protect mice from endotoxin-induced attenuation of HPV. Because a second receptor for LTβ has been recently identified, we also studied LTA4 hydrolase–deficient mice and found that endotoxin challenge also impaired HPV in LTA4 hydrolase–deficient mice. Because BLT1 appears to mediate most of the inflammatory response in mice and LTβ4, and LTA4 hydrolase-deficient mice do not produce LTβ4, it is unlikely that endotoxin-induced attenuation of HPV is mediated by LTβ4. In contrast, we found that pretreatment with MK571, a selective cysLT1 antagonist, preserved HPV in endotoxin-challenged wild-type mice. These results suggest that cysLTs play an important role in endotoxin-induced attenuation of murine HPV via a cysLT1-dependent mechanism. Furthermore, because HPV in endotoxin-challenged wild-type mice pretreated with MK571 was as robust as HPV in endotoxin-challenged 5-LO–deficient mice, it is likely that the protection of HPV in endotoxin-challenged 5-LO–deficient mice is largely attributable to the absence of cysLT production.

Lung MPO activity was increased after endotoxin challenge in wild-type mice, but the increase was attenuated in endotoxin-challenged 5-LO–deficient mice. We also noted that endotoxin did not cause leukopenia in endotoxin-challenged 5-LO–deficient mice, suggesting that fewer leukocytes were recruited into extravascular tissues or sequestered elsewhere. In contrast, pretreatment with MK571 did not attenuate the endotoxin-induced increase of lung MPO activity in wild-type mice. Although cysLT1 receptor mRNA has been detected in peripheral blood leukocytes as well as in smooth muscle cells of the lung, the role of cysLTs on leukocyte function is incompletely understood. It has been suggested that cysLTs possess important leukocyte-activating properties. It is therefore conceivable that the protective effects of cysLT1 receptor antagonism against endotoxin-induced attenuation of HPV are partly mediated by opposing the actions of cysLTs on leukocytes, as well as on other targets such as pulmonary vascular smooth muscle cells.

The attenuation of HPV in wild-type mice 22 hours after endotoxin challenge is not attributable to increased pulmonary cysLT 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 cysLT1 receptor antagonists, to prevent the attenuation of HPV in patients with clinical sepsis.

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