Leukotriene B$_4$ Activates Pulmonary Artery Adventitial Fibroblasts in Pulmonary Hypertension


Abstract—A recent study demonstrated a significant role for leukotriene B$_4$ (LTB$_4$) causing pulmonary vascular remodeling in pulmonary arterial hypertension. LTB$_4$ was found to directly injure luminal endothelial cells and promote growth of the smooth muscle cell layer of pulmonary arterioles. The purpose of this study was to determine the effects of LTB$_4$ on the pulmonary adventitial layer, largely composed of fibroblasts. Here, we demonstrate that LTB$_4$ enhanced human pulmonary artery adventitial fibroblast proliferation, migration, and differentiation in a dose-dependent manner through its cognate G-protein–coupled receptor, BLT1. LTB$_4$ activated human pulmonary artery adventitial fibroblast by upregulating p38 mitogen-activated protein kinase as well as Nox4-signaling pathways. In an autoimmune model of pulmonary hypertension, inhibition of these pathways blocked perivascular inflammation, decreased Nox4 expression, reduced reactive oxygen species production, reversed arteriolar adventitial fibroblast activation, and attenuated pulmonary hypertension development. This study uncovers a novel mechanism by which LTB$_4$ further promotes pulmonary arterial hypertension pathogenesis, beyond its established effects on endothelial and smooth muscle cells, by activating adventitial fibroblasts. (Hypertension. 2015;66:00-00. DOI: 10.1161/HYPERTENSIONAHA.115.06370.)

Key Words: fibroblasts ■ inflammation ■ leukotriene B$_4$ ■ NADPH oxidase ■ p38 mitogen-activated protein kinases ■ pulmonary artery ■ vascular remodeling

Pulmonary arterial hypertension (PAH) is a life-threatening disease associated with a wide variety of disorders. Current concepts of disease pathogenesis invoke a variety of factors, including vasoconstriction, metabolic derangement, BMP2 dysregulation, and inflammation that work in concert to produce serious pulmonary vascular pathology. The majority of patients with group I PAH exhibit evidence of systemic inflammation polarized toward Th1/Th17 or Th2 immunity depending on the underlying cause. Emerging evidence suggests that innate immunity contributes to disease development in certain forms of the condition, including PAH associated with connective tissue diseases; the degree of perivascular macrophage infiltration has been shown to correlate directly with vascular pathology and deranged hemodynamics.

In PAH, remodeling of small-to-medium-sized pulmonary arterioles is characterized by changes in all 3 layers of the vascular wall, including the intimal endothelial cells, medial smooth muscle cells, and adventitial fibroblasts. An inside-out response is a widely accepted concept of pulmonary vascular remodeling, in which infiltration of various inflammatory cells induce endothelial apoptosis and promote growth of the smooth muscle cell layer. However, recent studies strongly support an outside-in mechanism, in which adventitial fibroblasts serve as a source of pathological stimuli permeating the vascular wall. Vascular adventitial fibroblasts in this outer layer are activated by a variety of pathways that result in heightened proliferative, migratory, fibrotic, and inflammatory activity, including p38 mitogen-activated protein kinases (MAPKs) and NADPH oxidase 4 (Nox4).

The p38 MAPK pathway plays a pivotal role in numerous cellular functions. Elevated p38 MAPK activity contributes to hypoxia-induced pulmonary artery fibroblast proliferation. Similarly, increased expression of Nox4, in the absence of other stimuli, is sufficient to induce human...
pulmonary artery adventitial fibroblast (HPAAF) proliferation and migration. Nox is one of the major sources of cellular reactive oxygen species (ROS) known to be pathogenic in PAH. Interaction between the p38 MAPK and the Nox4-signaling pathways has been proposed for other diseases. However, whether activation of p38 MAPK and Nox4 pathways in fibroblasts are related to perivascular inflammation observed in the pulmonary adventitial transformation of PAH remains unknown. Our recent study demonstrated that the inflammatory mediator, leukotriene B\(_4\) (LTB\(_4\)), directly mediated some of the pathological changes observed in the inner and medial layers of pulmonary arterioles and raised the possibility that this molecule could affect pulmonary arteriolar adventitial cells as well.

LTB\(_4\) is one of a group of leukotrienes, lipid mediators produced from arachidonic acid metabolism through the 5-lipoxygenase (5-LO) pathway. In concert with 5-LO-activating protein, converts arachidonic acid to LTA\(_4\), LTA\(_4\) is then either hydrolyzed by LTA\(_4\) hydrolase to form LTB\(_4\), or is conjugated with reduced glutathione by LTC\(_4\) synthase to yield LTC\(_4\). Leukotrienes, especially LTB\(_4\), are implicated in several inflammatory diseases, including asthma, atherosclerosis, stroke, and myocardial infarction. In an animal model of autoimmune PAH, we discovered that LTB\(_4\) was significantly elevated in the bronchoalveolar lavage fluid of pulmonary hypertensive animals. We previously found that LTB\(_4\) levels were elevated significantly in patients with PAH, especially in those with connective tissue disorders; these latter individuals exhibited mean LTB\(_4\) levels 5-fold higher than those in healthy controls. By distinction, 6 of 8 patients with idiopathic PAH in that study exhibited normal LTB\(_4\) levels (a large scale study of LTB\(_4\) serum levels is currently being undertaken to more definitively assess this leukotriene in all group I PAH conditions). We demonstrated that LTB\(_4\) induced endothelial cell apoptosis and promoted smooth muscle cell proliferation in vitro. Blocking LTB\(_4\) production or LTB\(_4\)-mediated signaling reversed established severe pulmonary hypertension (PH) in addition to restoring remodeled pulmonary vasculature to patency. Given the strong role of LTB\(_4\) in this model of severe PAH, we investigated whether LTB\(_4\) also play a role in the phenotypic adventitial changes observed in PAH.

**Materials and Methods**

**Animal Model**

All in vivo experimental studies were approved by the Veterans Affairs Palo Alto Animal Care and Use Committee. Six- to eight-week-old athymic nude rats (nu/nu; Charles River Laboratories) were injected subcutaneously with a single dose of either SU5416 (SU, 10 mg/kg) dissolved in DMSO or DMSO (vehicle) alone. SU5416 is a small molecule inhibitor of the cytoplasmic tyrosine kinase segment of the vascular endothelial growth factor receptors flt and KDR (vascular endothelial growth factor receptor R1 and vascular endothelial growth factor receptor 2) and alone is sufficient to induce PH in athymic rats (by convention, animal models of PAH are referred to as PH). All animals were maintained in normoxic conditions. Bestatin, an LTA\(_4\) hydrolase inhibitor, was given orally at a dose of 1 mg/kg daily starting at the time of SU5416 administration. The p38 MAPK inhibitor (SB203580) was injected at a dose of 4 mg/kg IP 3x per week starting at the time of SU5416 injection. The dose was based on similar dosing regimens in previous in vivo studies.

**Human Plasma LTB\(_4\) Measurements**

The study was approved by the Institutional Review Board (IRB) at Stanford University with appropriate informed consent. Serum from deidentified healthy controls or patients with systemic sclerosis (SSc)-PAH was obtained from the IRB-approved Stanford Pulmonary Hypertension Biobank. LTB\(_4\) concentration was then determined by using the LTB\(_4\) enzyme immunoassay kit (Cayman Chemical) according to the manufacturer’s protocol.

**Human N-Terminal Pro-Brain Natriuretic Peptide Measurements**

Blood samples were collected at study entry by venipuncture in tubes containing EDTA. Blood samples were centrifuged at 3500g for 10 minutes at 4°C immediately after collection, and plasma samples were stored at –70°C. N-terminal pro-brain natriuretic peptide (ECLIA Elexys 2010 analyzer; Roche Diagnostics) were measured by commercially available assays in plasma samples never thawed before. The intra-assay coefficient of variation was 2.9% and the interassay coefficient of variation was 3.6%.

**Immunohistochemistry of Human Lung Tissue**

Paraffin-embedded, formalin-fixed human lung tissues from 2 healthy control subjects and 2 patients with SSc-PAH were obtained from the Pulmonary Hypertension Breakthrough Initiative Tissue Bank at Stanford. Antigen retrieval was performed by steaming the slides for 45 minutes using the IHC TekTM Epitope Retrieval System and then blocked with 1% donkey serum for 1 hour. The slides were incubated with anti–5-LO (Cell Signaling Technology), anti–CD68 (Dako), or anti–S100A4 (LifeSpan Biosciences Inc) in 1% donkey serum overnight at 4°C, followed by antirabbit Alexa Fluor 488 (Invitrogen) and antimouse Alexa Fluor 594 (Invitrogen) for 1 hour at room temperature. Images were acquired using a Zeiss 700 confocal microscope and analyzed with ImageJ.

**Immunohistochemistry of Rat Lung Tissue**

Lung samples were snap-frozen in OCT solution and were stored at –80°C. The following antibodies were used for immunohistochemistry: anti–5-LO (1:50, Cell Signaling Technology), anti–Nox4 (1:25, Abcam), antivimentin (1:20, Abcam), and anti–CD90 (1:200, Abcam). Images were analyzed with ImageJ blindly by 2 evaluators (X.J and W.T.). The adventitial compartment of the pulmonary vessels was determined by S100A4, vimentin, or CD90 staining. 5-LO–mediated signaling reversed established severe pulmonary hypertension (PH) in addition to restoring remodeled pulmonary vasculature to patency. Given the strong role of LTB\(_4\) in this model of severe PAH, we investigated whether LTB\(_4\) also play a role in the phenotypic adventitial changes observed in PAH.

**Cell Culture**

HPAAs were purchased from ScienCell and were grown in Fibroblast Medium (ScienCell); this media consisted of 2% fetal bovine serum, ascorbic acid, EDTA, and insulin. The study was approved by the Institutional Review Board (IRB) at Stanford University with appropriate informed consent. Serum from deidentified healthy controls or patients with systemic sclerosis (SSc)-PAH was obtained from the IRB-approved Stanford Pulmonary Hypertension Biobank. LTB\(_4\) concentration was then determined by using the LTB\(_4\) enzyme immunoassay kit (Cayman Chemical) according to the manufacturer’s protocol.

**Amplex Red Assay**

Hydrogen peroxide (H\(_2\)O\(_2\)) measurements in cells were made using the horseradish peroxidase–linked Amplex Red fluorescence assay.
Cells were incubated with 50-μmol/L Amplex Red (Invitrogen) and 0.125 U/mL horseradish peroxidase (Sigma) at 37°C for 10 minutes. Fluorescence readings were made at an excitation wavelength of 544 nm and an emission detection wavelength of 590 nm. Relative fluorescent units were calculated after subtraction of control groups (with catalase).

**Western Blots**

Cells were washed with ice-cold phosphate-buffered saline and lysed in radioimmunoprecipitation lysis and extraction buffer (Thermo Scientific) with a Halt protease inhibitor and phosphatase inhibitor cocktail (Thermo Scientific). Supernatants were collected and protein concentration was determined by the bicinchoninic acid assay (Thermo Scientific). Equal amounts of protein were size fractionated by 10% SDS–polyacrylamide gel and immunoblotted with corresponding antibodies.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide Assay**

Cell proliferation was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma). After synchronization for 24 hours by serum starvation, cells were treated with LTB₄ and inhibitors for 72 hours; 5 mg/mL of MTT were added and incubated for 4 hours. Solubilization solution was added to dissolve the MTT formazan crystals. Absorbance was spectrophotometrically measured at a wavelength of 570 nm with background subtraction at 690 nm.

**Bromodeoxyuridine Assay**

Bromodeoxyuridine (BrdU) Cell Proliferation Assay kit (Cell Signaling) was used to quantify cell proliferation. BrdU was added at last 12 hours. Absorbance was read at 450 nm.

**Migration Assay**

Cell migration was performed using a Boyden Chamber assay with 24 well, 8-μm pore size membrane invasion chambers (Fisher). Cells were synchronized with serum starvation, and 2×10⁴ cells were seeded into the upper chamber of the transwell, LTB₄, and inhibitors were added to the lower chamber. Membranes with migrated cells were fixed with methanol and stained with hematoxylin and eosin for 12 hours. The mean number of cells in five 10× random fields was used for quantification analysis.

**Measurements of Hemodynamics**

Rats were anesthetized with ketamine hydrochloride (70 mg/kg) and xylazine (10 mg/kg). Right ventricular (RV) systolic pressure measurements were obtained through the jugular vein into the RV using Micro Tip pressure transducer (model SPR-671, 1.4F; Millar Instruments). Signals were recorded continuously with a TC-510 pressure control unit 236927/R17 (Millar Instruments) coupled to a Bridge Amp (AD Instruments). Data were collected with the Powerlab7 data acquisition system (AD Instruments) and analyzed with Chart Pro software (AD Instruments). The RV was dissected from the left ventricle and septum. The Fulton index of RV/(left ventricle+septum) was calculated using the weight of RV, left ventricle, and septum to determine the degree of RV hypertrophy.

**Echocardiography**

Echocardiography evaluation of RV dimensions and pulmonary hemodynamics were performed using the Vivid 7 Dimension Cardiovascular Imaging System (GE), equipped with a 14-MHz transducer. Rats were lightly sedated with isoflurane for the duration of the procedure. The chests were depilated and the rats were laid supine on a warming handling platform. Pulmonary artery doppler tracings were obtained from the pulmonary artery parasternal short-axis view. The RV free wall was imaged from a modified parasternal long-axis view. All measurements were made in the expiratory phase of the respiratory cycle.

**Statistical Analysis**

GraphPad Prism version 5.0c was used for statistical analysis. With normally distributed data, unpaired t tests were applied for comparison of 2 groups. One-way ANOVA was used to compare multigroups. Differences between various groups at multiple time points were compared using 2-way ANOVA with Bonferroni multiple comparisons test for post hoc analyses. For comparisons between multiple experimental groups at a single time point, the Kruskall–Wallis test followed by Dunn multiple comparisons test for post hoc analyses was used. All data were represented as mean±SEM (SEM), and a P<0.05 was considered significant.

**Results**

**5-LO/LTB₄ Signaling Was Increased Around the Pulmonary Vascular Adventitia in PAH**

We previously evaluated the action of LTB₄ on cells within the intima and media⁴⁰ and now sought to assess its effects on the adventitia, where vascular inflammation is most prominent in PAH. The pulmonary arteriolar adventitial space is chiefly composed of fibroblasts and has recently been shown to induce a distinct proinflammatory/profibrotic macrophage phenotype in PH.⁴⁰ Because patients with SSc are prone to developing PAH and are known to have persistently activated fibroblasts,¹⁴⁻¹⁶ we assessed LTB₄ production in SSc-PAH blood and tissue. LTB₄ levels were significantly elevated in patients with SSc-PAH. These individuals exhibited mean LTB₄ levels 10-fold higher than that of the control group (Figure 1A and 1B). There was no significant correlation between serum LTB₄ levels and N-terminal pro-brain natriuretic peptide levels. 5-LO⁺ cells were noted in close proximity to the adventitial fibroblasts, stained with S100A4 (also called fibroblast-specific protein 1), in SSc-PAH compared with controls (Figure 1C and 1D). Approximately 70% of 5-LO⁺ cells observed in the thickened adventitia were CD68⁺ macrophages (Figure S1A and S1B). In diseased PH lungs, containing of 5-LO and S100A4 was also observed in the intimal cells of occluded vessels suggesting an activation of LTB₄ biosynthetic machinery in these cells.

In a model of autoimmune PAH,¹⁶ enhanced 5-LO expression was observed either adjacent to or colocalized with adventitial fibroblasts, stained with vimentin, from SUs416-treated athymic rat lungs (Figure 1E and 1F). LTB₄ synthesis inhibition with bestatin, an intervention that both prevents and reverses experimental PH, prevented adventitial remodeling and also mitigated 5-LO expression.¹⁶ These cumulative results suggest that increased LTB₄ biosynthesis is related to the expansion of vascular adventitial fibroblasts in PH.

**LTB₄ Promoted Proliferation, Migration, and Differentiation of HPAAFs**

During the pathological remodeling process of PH, PAAFs show enhanced proliferation, migration, and differentiation.¹⁵⁻¹⁷ To determine whether LTB₄ itself promotes HPAAF proliferation in the absence of other stimuli, we first monitored HPAAF growth in response to various physiologically relevant concentrations of LTB₄ using the MTT assay, cell counting, and BrdU assay (Figure 2A–2C). LTB₄ promoted HPAAF proliferation in a dose-dependent manner. By contrast, when lung parenchymal fibroblasts were cultured in the same LTB₄ conditions, no significant changes in cell
Figure 1. Five-lipoxygenase (LO)/leukotriene B$_4$ (LTB$_4$) signaling is increased around pulmonary vascular adventitial fibroblasts in pulmonary arterial hypertension (PAH). A, Plasma LTB$_4$ concentration in 10 healthy controls and 10 patients with systemic sclerosis (SSc)-PAH. B, Demography table. C, Representative immunofluorescence images of human lung sections stained with 5-LO (green) and S100A4 (fibroblasts, red) from healthy individuals and patients with SSc-PAH. D, Morphometric analysis of images in C. Number of 5-LO$^+$ cells within 5 $\mu$m of the pulmonary adventitia. E, Representative immunofluorescence images of lung sections stained with 5-LO (green) and Vimentin (fibroblasts, red) from dimethyl sulfoxide (DMSO; negative control), SU (pulmonary hypertension), or SU+bestatin (Continued)
proliferation were detected (Figure S2), a finding suggesting that the response to LTB₄ is cell type dependent. Western blot analysis of HPAAFs demonstrated increased expression of the proliferating cell nuclear antigen in LTB₄-exposed HPAAFs consistent with cell growth (Figure 2D).

To further assess LTB₄ effects on HPAAFs, in vitro migration was evaluated using a Boyden Chamber assay, which showed that the promotion of in vitro migration was concentration dependent (Figure 2E and 2F). Because the differentiation of HPAAFs into collagen-producing, α-smooth muscle actin–expressing myofibroblasts is critical for vascular stiffness, contractility, and angiogenesis in PAH, we investigated effects of LTB₄ on myofibroblast differentiation. LTB₄ promoted myofibroblast differentiation in a concentration-dependent manner (Figure 2G). Next, because p38 MAPK also mediates the proliferation and migration of PAAFs, but not systemic artery fibroblasts, we tested whether LTB₄ promoted HPAF activation through p38 MAPK signaling. Western blots show increased phosphorylation of p38 MAPK in the presence of LTB₄ consistent with LTB₄ activation of p38 MAPK signaling in HPAAFs (Figure 2H). These data collectively demonstrate, for the first time, that LTB₄ mediates HPAF proliferation, migration, and differentiation and concomitantly activates signaling via p38 MAPK.

**LTB₄-Mediated HPAF Proliferation, Migration, and Differentiation Required BLT1 Receptor Signaling and p38MAPK Pathway Activation**

To further investigate the molecular mechanism by which LTB₄ induces HPAF activation, we used the BLT1 antagonist

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Leukotriene B₄ (LTB₄) promotes proliferation, migration, and differentiation in human pulmonary artery adventitial fibroblast (HPAAF). Proliferation of HPAAF with increasing doses of LTB₄ treatment for 72 hours was measured by (A) the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, (B) cell counting, and (C) bromodeoxyuridine (BrdU) assay. Data are presented as mean±SEM (⁎P<0.05). **D, E,** Proliferating cell nuclear antigen (PCNA) expression was measured by Western blot after treatment with LTB₄ for 24 hours. **E and F,** Migration of HPAAF after LTB₄ exposure were determined and quantified by Boyden Chamber assay. Data are presented as mean±SEM (⁎P<0.05). **G and H,** α-smooth muscle actin (SMA) and p-p38 mitogen-activated protein kinase expression after LTB₄ treatment for 24 hours as determined by Western blot. β-actin was used as a loading control. The experiments were repeated 3×.
(U75302) and p38 MAPK inhibitor (SB203580) in HPAAF cultures. U75302 (1 μmol/L) reversed the proliferative HPAAF activity of LTB₄ as determined by the MTT assay, cell counting, BrdU assay, and proliferating cell nuclear antigen expression (Figure 3A–3D). Similarly, treatment with SB203580 (10 μmol/L) inhibited LTB₄-induced cell proliferation (Figure 3A–3C; Figure S3). Western blot analysis of phosphorylated p38 MAPK protein levels showed that LTB₄-mediated p38 MAPK activation was dampened by BLT1 blockade (Figure 3D; increased p38 phosphorylation in the SB203580-treated group is probably attributable to the fact that this agent inhibits p38 catalytic activity by binding to the ATP-binding pocket without affecting phosphorylation of p38 by upstream kinases). Collectively, these data suggest that LTB₄ controls HPAAF growth through engagement of its high affinity receptor, BLT1, and through activation of p38 MAPK. Inhibition of p38 MAPK signaling by pretreating cells with SB203580 limited LTB₄-mediated fibroblast migration and fibroblast to myofibroblast transformation (Figure 3E–3G).

![MTT assay](image1)

![Cell counting](image2)

![BrdU assay](image3)

![PCNA](image4)

![p-p38](image5)

![p38](image6)

![β-Actin](image7)

![Control](image8)

![LTB4](image9)

![LTB4+U75302](image10)

![LTB4+SB203580](image11)

Figure 3. Leukotriene B₄ (LTB₄)-induced human pulmonary artery adventitial fibroblast (HPAAF) proliferation, migration, and differentiation were inhibited by pretreatment with BLT1 blockade (U75302) or p38 mitogen-activated protein kinase (MAPK) inhibition (SB203580). HPAAF proliferation (A), cell counting (B), bromodeoxyuridine (BrdU) assay (C), proliferating cell nuclear antigen (PCNA) expression (D), migration (E and F), and differentiation (G) were determined after pretreatment of LTB₄ receptor antagonist U75302 (1 μmol/L) or p38 MAPK inhibitor SB203580 (10 μmol/L) in the presence of LTB₄. #According to the manufacturer, increased p38 phosphorylation in the SB203580-treated group in D is likely attributable to the fact that this agent inhibits p38 catalytic activity by binding to the ATP-binding pocket without affecting phosphorylation of p38 by upstream kinases. Data are presented as means±SEM. (*P<0.05). The experiments were repeated 3×. MTT indicates 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; and SMA, smooth muscle actin.
Blocking p38 MAPK Signaling Attenuated Experimental Autoimmune PH

Because LTB₄-dependent activation of p38 MAPK pathway was strongly implicated as a critical mediator of PAAFs in autoimmune PH and because p38 MAPK phosphorylates 5-LO in polymorphonuclear leukocytes and causes a 4-fold increase in 5-LO activity, we hypothesized that inhibiting p38 MAPK signaling could have protective effects in this model of autoimmune PH. SB203580 dosing was initiated at the time of SU5416 administration in athymic rats. After SU5416 administration, echocardiographic evidence of PH was first detected between weeks 1 and 2 and severe PH was evident by week 3. Invasive hemodynamic measures were taken at 3 weeks after SU5416 administration. In the SU5416-athymic PH rats treated with SB203580, serial echocardiography showed that PH was attenuated. Interval improvement is manifested by decreasing RV wall thickness and longer pulmonary artery acceleration times (Figure 4A and 4B). Accordingly, reduced RV systolic pressure and RV hypertrophy were observed with SB203580 treatment (Figure 4C and 4D). Improvement in mortality was found 3 weeks after SB203580 treatment, indicating that p38 MAPK inhibition treatment is effective in attenuating PH progression and mortality in this model of autoimmune PAH (Figure 4E).

Figure 4. Blocking p38 mitogen-activated protein kinase (MAPK) signaling attenuates experimental pulmonary hypertension. Rats were treated with the p38 MAPK inhibition (SB203580) starting at the time of SU administration. A and B, Animals were monitored by echocardiography weekly. C and D, Hemodynamic measurements were done at week 3. Right ventricular systolic pressure (RVSP) measurements in dimethyl sulfoxide (DMSO), SU, and p38 MAPK inhibition treatment groups were assessed at week 3 post SU. RV hypertrophy measurements as assessed by the RV/left ventricle (LV)+septum (S) weight ratios. E, Survival of rats after treatment was compared with DMSO and SU rats (n=6 per group). Data are expressed as mean±SEM (*P<0.05). The experiments were repeated 3×. PAAT indicates pulmonary artery acceleration time.
Blocking LTB₄ Synthesis and Inhibiting p38 MAPK Signaling Decreased Fibroblast Expansion and Nox4 Expression in Experimental PH

Globally, inhibiting p38 MAPK signaling with SB203580 treatment also decreased LTB₄ levels in bronchoalveolar lavage fluid and reduced 5-LO expression associated with the thickening of adventitial fibroblast layer in PH (Figure 5A and 5B). Oxidative stress is widely associated with vascular injury because of an excessive production of ROS, which can act through p38 MAPK to affect cellular changes. Elevated ROS lead to vascular remodeling and increased PA pressures in PH. Nox is one of the major sources of cellular ROS known to be pathogenic in PAH, and Nox4 is the most important isoform of Nox in adventitial fibroblasts. Nox4-positive fibroblasts were increased in PH lungs (Figure 5B) as were Nox4/5-LO double-positive fibroblasts. Both bestatin and SB203580 treatments significantly reduced Nox4 expression in PAAFs and decreased the thickening of pulmonary arteries. (Figure 5B; Figure S5).

We next investigated whether blocking LTB₄ biosynthesis with bestatin or inhibiting p38 MAPK signaling with SB203580 could also reduce tissue H₂O₂ levels in PH lungs. Tissue H₂O₂ production was measured using horseradish peroxidase–linked Amplex Red. Increased Amplex Red fluorescence correlates with elevated Nox4 expression in the lungs. Both bestatin and SB203580 treatments effectively reduced Nox4-related H₂O₂ production (Figure 5C).

LTB₄-Induced Nox4 Expression, Hydrogen Peroxide Production, and Activation of HPAAFs

Because upregulation of Nox4 and hydrogen peroxide (H₂O₂) in proliferating pulmonary adventitial fibroblasts has been noted in developing PH, we next investigated whether Nox4 and H₂O₂ production in fibroblasts were increased by LTB₄. HPAAFs treated with LTB₄ lead to an elevated Nox4 expression with a corresponding increased H₂O₂ production in a concentration-dependent manner (Figure 6A and 6B). H₂O₂ production was inhibited by blocking BLT1, p38 MAPK, and Nox4 (Figure 6C). As shown in Figure 6D, increased p38 phosphorylation in the SB203580-treated group is probably because of this agent inhibiting p38 catalytic activity without affecting phosphorylation of p38 as described in Figure 3B. Inhibition of Nox4 decreased activation of p38 MAPK and inhibition of p38 MAPK also inhibited Nox4 expression (Figure 6D). These results suggest that a feedforward system in which ROS can activate p38 MAPK, and that p38 MAPK can subsequently upregulate Nox4 expression and ROS production in HPAAFs. Furthermore, proliferation, migration, and differentiation of HPAAF, induced by LTB₄, were reversed by Nox4 inhibition (Figure 6E–6K; Figure S3). Cumulatively, these results strongly support an important role for LTB₄ and its receptor BLT1 in the activation of HPAAFs via the p38 MAPK pathway and acting in concert with the ROS molecule, Nox4. (Figure 7).

Discussion

We recently described an important role for the increased expression of macrophage-derived LTB₄ in PH, showing how this eicosanoid specifically induces PA endothelial apoptosis and smooth muscle cell proliferation. Pathological events strongly implicated in the pathogenesis of PAH. Unexplored in this previous study was a role for LTB₄ in the third outer layer of the affected arterioles, the adventitia. This outer vascular zone is where abundant LTB₄-secreting macrophages are chiefly observed. In this study, we uncovered a novel function of LTB₄ specifically the activation of HPAAFs. Interestingly, this effect was not observed in nonvascular lung fibroblasts. The possibility that other fibroblast populations would not proliferate in response to LTB₄ was suggested by a previous study, in which a leukotriene-blocking 5-LO–activating protein inhibitor had no effect on serum-induced growth on the National Institutes of Health/3T3 cell line (mouse embryonic fibroblast cell line). We demonstrated that LTB₄-induced HPAAF proliferation, migration, and differentiation through p38 MAPK activation and upregulation of Nox4. Blocking LTB₄ signaling through its cognate high-affinity heterotrimetric G-protein–coupled receptor, BLT1, inhibited LTB₄-mediated proliferation, migration, and differentiation of HPAAF. Blocking p38 or Nox4 did the same, demonstrating a linked pathway. In the context of our previous study, LTB₄ seems to be an important inflammatory mediator, which is highly expressed at the site of disease activity in PAH, which is uniquely capable of modulating activation of a variety of cell types culminating in remodeling of the entire vascular wall.

We confirmed that diseased pulmonary arteries are surrounded by a large population of 5-LO⁺ cells, which are mostly CD68⁺ macrophages that are in close proximity to adventitial fibroblasts. Some adventitial fibroblasts and cells within the occluded vascular lumens of the PAH lung tissue were 5-LO⁺, suggesting that there is an aberrantly active LTB₄ biosynthetic machinery localized in these cells. Previous studies suggest that S100A4⁺ endothelial cells are involved in tumor angiogenesis. The coexpression of S100A4 and 5-LO in the intimal lumen also suggests an association between the LTB₄ signaling and the occlusive intimal remodeling of PAH. PAH, especially SSc-PAH, is characterized by robust fibroproliferative changes in pulmonary arteries. PAAFs undergo significant phenotypic changes characterized by increased proliferative, migratory, fibrotic, and inflammatory activity. These phenotypic changes have been demonstrated to mediate macrophage-associated inflammation that influence blood vessel tone and cause vascular remodeling. The sequence of events leading to PAAF activation is not fully understood, but evidence suggests that activation of p38 MAPK and increased expression of Nox4 in the adventitia may contribute to the altered fibroblast behavior.

As a member of the MAPK family, p38 MAPK is a critically important signaling pathway affecting inflammation, shear stress, and hypoxia. Pharmacological inhibition of p38 MAPK increases nitric oxide generation, reduces superoxide anion burden, and restores hypoxia-induced endothelial dysfunction in rats with hypoxia-induced PH. Here, we demonstrated that the LTB₄-induced activation of HPAAF is p38 MAPK dependent. Because p38 MAPK catalyzes the phosphorylation of 5-LO at the Ser271 site, p38 MAPK regulates LTB₄ production in leukocytes at the enzymatic post-translational level. Our data support the concept of a positive
Figure 5. Blocking leukotriene B$_4$ (LTB$_4$) synthesis and inhibiting p38 mitogen-activated protein kinase signaling decreases fibroblast activation and Nox4 in experimental pulmonary arterial hypertension. A, LTB$_4$ concentrations in the bronchoalveolar lavage fluid of dimethyl sulfoxide (DMSO), SU, or SU+SB203580 animals. B, Immunofluorescence images of rat lung tissues stained with 5-lipoxygenase (LO; magenta), Vimentin (green), and Nox4 (red). Yellow-dashed lines approximate the adventitial zone. C, Representative immunofluorescence images of lung sections stained with Nox4 (green). Amplex Red (red) indicates tissue H$_2$O$_2$ level. 4',6-diamidino-2-phenylindole (DAPI; blue) stains nuclei and differential interference contrast (DIC) highlights alveolar and vascular structures; n=5. Data are expressed as mean±SEM (*P<0.05).
Figure 6. Leukotriene B4 (LTB4)–induced Nox4 expression, hydrogen peroxide (H2O2) production, and human pulmonary artery adventitial fibroblast (HPAAF) activation. A, HPAAFs were treated with LTB4 at concentrations of 200 and 400 nM; H2O2 production was measured by Amplex assay. B, Nox4 expression was determined by Western blot. C, After pretreatment with U75302 (1 μmol/L), SB203580 (10 μmol/L), or the Nox4 inhibitor apocynin (300 μmol/L), the effects of exogenous LTB4 on HPAAFs were assayed. H2O2 production was measured by the Amplex assay. D, Western blots were used to determine the expression of Nox4, p-p38 mitogen-activated protein kinase (MAPK), and total p38 MAPK. β-actin was used as a loading control. E–G, MTT assay (E), cell counting (F), bromodeoxyuridine (BrdU) assay (G), proliferating cell nuclear antigen (PCNA) expression (H), migration (I and J), and differentiation (K) were determined with Nox inhibition (apocynin) in the presence of LTB4. *Note again in D, increased p38 phosphorylation in the SB203580-treated group probably because of this agent inhibiting p38 catalytic activity without affecting phosphorylation of p38 as described in Figure 3D**; *P<0.05). The experiments were repeated 3×. SMA indicates smooth muscle actin.
feedback loop between LTB₄ and p38 MAPK in HPAAFs. In addition, in our rat model of autoimmune PH, inhibition of p38 MAPK with SB203580 attenuated LTB₄-associated perivascular inflammation, decreased the expression of Nox4, prevented structural changes in the arteriolar adventitia, and limited the development of PH. However, compared with the previously demonstrated effects of LTB₄ antagonism, the impact of p38 MAPK inhibition in vivo was relatively less effective. Importantly, LTB₄ inhibitors not only prevent PAH development (in contrast to the mild attenuation observed with the p38 MAPK inhibitor used in this study) but they also reverse established pulmonary vascular disease.

The modest effect of p38 inhibition in this autoimmune animal model probably reflects the pleiotropic nature of p38 MAPK activity in autoimmune PH. For example, interleukin-6, a cytokine implicated in PAH development and autoimmune disease, also inhibited p38 signaling in the development of PH in a mouse model suggesting that dampening this signaling cascade is associated with deleterious effects.

Beyond its myriad effects on inflammation, p38 inhibition also appeared to exert an effect on RV remodeling even before an effect on RV systolic pressure and pulmonary artery acceleration times was detected. When considered together with the improved survival observed in rats receiving this therapy, this finding suggested that this drug exerted a direct salubrious effect on the myocardium. Several studies have documented the cardioprotective properties of p38 inhibition during myocardial infarction and cardiac ischemia-reperfusion injury.

In addition to the pleiotropic nature of p38 signaling, another possibility for p38 inhibitor effects is that LTB₄ may be activating fibroblasts through additional previously implicated pathways, such as JNK (c-Jun N-terminal protein kinase) and PKC (protein kinase C). However, given that p38 MAPK inhibitors have now been demonstrated to have at least some effect in at least 3 preclinical models of PH, there is at least the possibility selective p38 inhibition may play some clinical role as an adjunctive treatment for certain PH conditions. Although early clinical studies of p38 MAPK inhibitors in autoimmune disease demonstrated poor efficacy and unacceptable side effects, there has been cautious optimism about the use of other p38 MAPK inhibitor compounds in chronic obstructive pulmonary disease and atherosclerosis.

Given the role of p38 MAPK in governing hypoxic stress in fibroblasts, we sought to evaluate the key source of ROS in PAH. Nox4, a member of the NADPH oxidase family, is a major intracellular source of ROS. Nox4 is key for mediating numerous cellular functions, including cellular proliferation, differentiation, migration, and apoptosis. Of the 5 Nox isoforms encoded by the human genome, 4 (Nox1, Nox2, Nox4, and Nox5) are expressed in vascular cells. In mice, genetic deletion of Nox2 has been shown to reverse hypoxia-initiated PH, and Nox1 has been shown to be important for systemic hypertension. However, it was recently discovered that only expression of the Nox4 isoform increases in rat models of PH and in human PH. Increased expression of Nox4 induces fibroblast proliferation and migration. Similarly, Nox4 inhibition reduces proliferation of fibroblasts that are isolated from the pulmonary arteries of monocrotaline-treated rats. Nox4 inhibitors effectively prevent monocrotaline-induced PH but are not therapeutically sufficient to halt the disease progression. Of relevance to this study, LTB₄ has been shown to induce Nox activation and ROS production in mammalian cells. Here, we confirmed that Nox4 expression is present mainly in pulmonary adventitial fibroblasts in the athymic rat model of experimental autoimmune PH. In addition, we documented that LTB₄ induces the protein expression of Nox4 in HPAAFs in a concentration-dependent manner, and that this expression correlates with increased p38 MAPK activity. In this study, inhibiting p38 MAPK decreased Nox4 expression. As given in previous studies that showed Nox4 overexpression causes p38 MAPK phosphorylation, p38 MAPK and Nox4 pathways are likely synergistic in the development of PAH.

In conclusion, LTB₄, which causes PA endothelial cell apoptosis and PA smooth muscle cell growth, also causes BLT1-dependent PAAF activation via p38 MAPK signaling in concert with Nox4 generation. Collectively, these findings emphasize the role of LTB₄ in the pathobiology of autoimmune PAH.

**Perspectives**

A microvasculopathy with an essential inflammatory component underlies PAH. The poor prognosis of patients afflicted by this disease despite treatment with the currently available vasodilator drugs makes the development of new treatment strategies imperative. LTB₄ can induce vascular inflammation in all 3 layers of pulmonary arterioles causing endothelial cell apoptosis, vascular smooth muscle cell, and fibroblast proliferation. LTB₄-directed therapeutic strategies seem to be justified and should be evaluated in autoimmune forms of severe PAH.

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**Disclosures**

W. Tian and M.R. Nicolls cofounded Eiccose, LLC, a company which is currently investigating the role of LTB₄ antagonism in clinical pulmonary arterial hypertension.

**References**


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Novelty and Significance

What Is New?
- Our studies demonstrate that the eicosanoid, leukotriene B₄ (LTB₄), activates pulmonary artery fibroblasts, a finding of potential relevance in the pathogenesis of pulmonary arterial hypertension.
- We also show that LTB₄ stimulates these cells by binding to its high affinity receptor, BLT1, on fibroblasts and through activation of p38 MAPK and Nox4.

What Is Relevant?
- LTB₄ is an important mediator of vascular inflammation in pulmonary arterial hypertension, acting on pulmonary artery endothelial, smooth muscle cells, and adventitial fibroblasts.

- Activated macrophages, which are actively secreting LTB₄, are concentrated in the outer adventitial layer of affected arteries and may help explain the link between inflammation and disease.

Summary

Results from this study show a novel mechanism by which LTB₄ facilitates pulmonary arterial hypertension, beyond its established effects on endothelial and smooth muscle cells, by activating adventitial fibroblasts.
LTB₄ activates pulmonary artery adventitial fibroblasts in pulmonary hypertension.

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**Figure S1.** 5-LO positive macrophages are concentrated around the adventitial compartment.

A, Representative immunofluorescence images of lung sections stained with 5-LO (green) and CD68 (red) from SU treated animals. B, 5-LO+ and CD68+ double positive cells were counted and grouped as cell around the adventitia or cells outside of the adventitia. n= 5; DAPI (blue) stains nuclei. Data are expressed as means ± SEM.
Figure S2. LTB4 has no effect on proliferation in human lung fibroblasts (HLF). HLF were treated with LTB4 for 72hrs. Proliferation of HLF was measured by A. MTT assay, B, cell counting, C, BrdU assay. Data are presented as mean ± SEM. (n.s = non-significant) The experiments were repeated three times.
**Figure S3.** Inhibitors have no toxicity on HPAAF. MTT assay were performed after 72 hrs with treatment of U75302 (1μM), SB203580 (10μM) or apocynin (300μM) in HPAAF. Data are presented as mean ± SEM. (n.s = non-significant) The experiments were repeated three times.
Figure S4. p38 MAPK inhibition treatment with SB203580 reduces lung inflammation in experimental PH.

The macrophage-associated cytokine TNF-α and chemokines, CXCR1 and CCR2 in PH were evaluated by RT-PCR of lung tissues (n=3 experiments per group). Data are expressed as means ± SEM. (*: p<0.05)
Figure S5. p38 MAPK inhibition therapy reduced Nox4 activation around the pulmonary vascular adventitia in experimental PH. Confocal images of rat lung tissues stained with Nox4 (green), CD90 (fibroblast, red) and 5-LO (magenta) from SB203580 treatment group; n=5. DAPI (blue) stains nuclei; DIC highlights alveolar and vascular structures.
Leukotriene B₄ Activates Pulmonary Artery Adventitial Fibroblasts in Pulmonary Hypertension


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