Pulmonary Arterial Hypertension

Leukotriene B₄ Activates Pulmonary Artery Adventitial Fibroblasts in Pulmonary Hypertension


Abstract—A recent study demonstrated a significant role for leukotriene B₄ (LTB₄) causing pulmonary vascular remodeling in pulmonary arterial hypertension. LTB₄ 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 LTB₄ on the pulmonary adventitial layer, largely composed of fibroblasts. Here, we demonstrate that LTB₄ enhanced human pulmonary artery adventitial fibroblast proliferation, migration, and differentiation in a dose-dependent manner through its cognate G-protein–coupled receptor, BLT1. LTB₄ 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 LTB₄ further promotes pulmonary arterial hypertension pathogenesis, beyond its established effects on endothelial and smooth muscle cells, by activating adventitial fibroblasts. (Hypertension. 2015;66:1227-1239. DOI: 10.1161/HYPERTENSIONAHA.115.06370.)

Key Words: fibroblasts • inflammation • leukotriene B₄ • 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.¹⁻⁵ Current concepts of disease pathogenesis invoke a variety of factors, including vasoconstriction, metabolic derangement, BMPR2 dysregulation, and inflammation that work in concert to produce serious pulmonary vascularopathy.⁶⁻⁸ 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.²¹ 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.⁷,⁹,¹⁰

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.¹¹ 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.¹² However, recent studies strongly support an outside-in mechanism, in which adventitial fibroblasts serve as a source of pathological stimuli permeating the vascular wall.¹²⁻¹⁵ 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).¹⁶⁻²³

The p38 MAPK pathway plays a pivotal role in numerous cellular functions.²⁴⁻²⁵ Elevated p38 MAPK activity contributes to hypoxia-induced pulmonary artery fibroblast proliferation.¹⁷⁻¹⁹,²⁶ Similarly, increased expression of Nox4, in the absence of other stimuli, is sufficient to induce human...

Received August 23, 2015; first decision September 5, 2015; revision accepted September 10, 2015.

From the VA Palo Alto Health Care System, Palo Alto, CA (J.Q., W.T., X.J., R.T., Y.K.S., E.M.S., A.B.T., M.R.N.); Department of Medicine, Division of Pulmonary and Critical Care Medicine (J.Q., W.T., X.J., R.T., Y.K.S., E.M.S., A.B.T., S.J., R.T.Z., M.R.N.); Department of Immunology and Rheumatology (A.V., D.F.F., L.C.), and Department of Pediatrics (M.R.), Stanford University School of Medicine, CA; Department of Internal Medicine, Victoria Johnson Center for Obstructive Lung Diseases and Pulmonary and Critical Care Medicine Division, Virginia Commonwealth University, Richmond (N.F.V.); Department of Internal Medicine, University of Michigan Health Systems, Ann Arbor (M.P.-G.); and Department of Pediatrics, Cardiovascular Pulmonary Research Laboratories, University of Colorado Denver School of Medicine, Aurora (K.R.S.).

* These authors contributed equally to this work.

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.115.06370/-/DC1.

Correspondence to Mark R. Nicolls, VA Palo Alto Health Care System, Stanford University School of Medicine, Med111P, 3801 Miranda Ave, Palo Alto, CA 94304. E-mail mnicolls@stanford.edu

© 2015 American Heart Association, Inc.

Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.115.06370

1227
pulmonary artery adventitial fibroblast (HPAAF) proliferation and migration.\(^{20}\) Nox is one of the major sources of cellular reactive oxygen species (ROS) known to be pathogenic in PAH.\(^{2,27–29}\) Interaction between the p38 MAPK and the Nox4-signaling pathways has been proposed for other diseases.\(^ {30}\) 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 arterial 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.\(^ {31}\) 5-LO, 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,\(^ {32}\) atherosclerosis,\(^ {33}\) stroke,\(^ {34}\) and myocardial infarction.\(^ {35}\) 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\(_4\) serum levels is currently being undertaken to more definitively assess this leukotriene in all group I PAH conditions.\(^ {36}\) 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 patentcy.\(^ {37}\) 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 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\(^ {38}\) (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 3× per week starting at the time of SU5416 injection. The dose was based on similar dosing regimens in previous in vivo studies.\(^ {37,38}\)

**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 (ECLA Elecsys 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 Steamer 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, BioGenex, 800 nM), U75302 (Cayman Chemical, 1 mol/L), SB203580 (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.

**Cell Culture**

HPAAFs were purchased from ScienCell and were grown in Fibroblast Medium (ScienCell); this media consisted of 2% fetal bovine serum, 1% fibroblast growth supplement, and 1% penicillin/streptomycin solution. Normal human lung fibroblasts were purchased from ATCC (LL24, ATCC CCL-15), and were grown in DMEM (Life Technologies) containing 10% fetal bovine serum and 1% penicillin/streptomycin solution. Cells were grown at 37°C in 5% CO\(_2\) incubator and used between passages 3 and 8. LTB\(_4\) (Cayman Chemical, 200, 400, 800 nM), U75302 (Cayman Chemical, 1 μmol/L), SB203580 (Cell signaling, 10 μmol/L), and apocynin (Sigma, 300 μmol/L) were used for treatment of cells.

**Amplex Red Assay**

Hydrogen peroxide (H\(_2\)O\(_2\)) measurements in cells were made using the horseradish peroxidase–linked Amplex Red fluorescence assay.\(^ {39}\)
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 2×10⁵ 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 media¹⁰ 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,¹¹,¹³–¹⁵ 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,¹⁰ enhanced 5-LO expression was observed either adjacent to or colocalized with adventitial fibroblasts, stained with vimentin, from SU5416-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.¹⁰ 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, PAAF show enhanced proliferation, migration, and differentiation.¹₂–¹⁴ 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 B4 (LTB4) signaling is increased around pulmonary vascular adventitial fibroblasts in pulmonary arterial hypertension (PAH). A, Plasma LTB4 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 μm 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,14,44 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,26 we tested whether LTB4 promoted HPAAF 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 HPAAF proliferation, migration, and differentiation and concomitantly activates signaling via p38 MAPK.

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

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

---

**Figure 1.** Continued (bestatin-treated) animals. F, Morphometric analysis of E. In A, data are presented in the scatter plots showing minimal to maximal values and all data points. In C and E, 4′,6-diamidino-2-phenylindole (blue) stains nuclei; differential interference contrast (DIC) highlights alveolar and vascular structures; n=5. Yellow-dashed lines indicate the adventitia area. In A, D, and F, data are presented as mean±SEM. (*P<0.05). NTproBNP indicates N-terminal pro-brain natriuretic peptide.

---

**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, 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×.
Hypertension December 2015

(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 (Figure 3D) 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. Data are presented as mean±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.

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 mean±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).

With p38i, the inflammatory markers (chemokine receptor CCR2, transforming growth factor-α, and fractalkine receptor CX3CR1) in lung tissue were also decreased (Figure S4).

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.
Both bestatin and SB203580 treatments effectively reduced fluorescence correlates with elevated Nox4 expression in the lungs. Oxidase–linked Amplex Red. Increased Amplex Red fluorescence correlates with elevated Nox4 expression in the lungs. Both bestatin and SB203580 treatments significantly reduced Nox4 expression with a corresponding increased H2O2 production in a forward system in which ROS can activate p38 MAPK, and increased expression of Nox4. Blocking LTB4 signaling through its cognate high-affinity heterotrimeric 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 arterioles 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 lumen 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

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, 2 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 heterotrimeric 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 our previous study, we demonstrated that the LTB4-induced activation of HPAAFs 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

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. 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 feed-forward 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. Two 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 heterotrimeric 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 arterioles 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 lumen 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 B4 (LTB₄) synthesis and inhibiting p38 mitogen-activated protein kinase signaling decreases fibroblast activation and Nox4 in experimental pulmonary arterial hypertension. A, LTB₄ 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 H₂O₂ 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. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (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,10 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.10

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.55,56 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.57-60

In addition to the pleiotropic nature of p38 signaling, another factor possibly mitigating 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).22,23 However, given that p38 MAPK inhibitors have now been demonstrated to have at least some effect in at least 3 preclinical models of PH,61 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.25

Given the role of p38 MAPK in governing hypoxic stress in fibroblasts, we sought to evaluate the key source of ROS in PH, Nox4, a member of the NADPH oxidase family, is a major intracellular source of ROS.28,29,62 Nox4 is key for mediating numerous cellular function, including cellular proliferation, differentiation, migration, and apoptosis.84 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 the 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.20 Of relevance to this study, LTB4 has been shown to induce Nox activation and ROS production in mammal cells.64,65 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 shown in previous studies that showed Nox4 overexpression causes p38 MAPK phosphorylation,65,66 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.

**Acknowledgments**

We acknowledge Dr Lajos Gera for the synthesis of SU5416 and Dr Lingli Wang for preparing the human tissue sections for histology.

**Sources of Funding**

This work was supported by National Institutes of Health grants HL125739, HL082662 (M.R. Nicolls), and NHLBI-HV-10-05 (M. Rabinovitch and M.R. Nicolls).

**Disclosures**

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

**References**


unique proliferative response in adventitial fibroblasts by activating
10.1093/cvr/cvs194.

3. Das M, Burns N, Wilson SJ, Zawada WM, Stenmark KR. Hypoxia exposure
induces the emergence of fibroblasts lacking replication repressor
signals of PKC zeta in the pulmonary artery adventitia. Cardiovasc Res.

4. Zawada WM, Stenmark KR, Das M. Hypoxia induces unique proliferative
response in adventitial fibroblasts by activating
10.1093/cvr/cvs194.

5. McLaughlin VV, Shah SJ, Souza R, Humbert M. Management of pulmo-

10.1016/j.jacc.2015.03.540.

6. Rabinovitch M, Guignabert C, Humbert M, Nicollis MR. Inflammation
and immunity in the pathogenesis of pulmonary arterial hypertension.

7. Guignabert C, Tu L, Girerd B, Ricard N, Huertas A, Montani D, Humbert M.
New molecular targets of pulmonary vascular remodeling in pulmo-
nary arterial hypertension: importance of endothelial communication.

8. Archer SL, Weir EK, Wilkins MR. Basic science of pulmonary arterial
hypertension for clinicians: new concepts and experimental

adventitia in pulmonary vascular remodeling. Physiol Monogr (Bethesda).

10. Stenmark KR, Nozik-Grayck E, Gerasimovskaya E, Anwar A, Li M,
Riddle S, Frid MG. The adventitia: essential role in pulmonary vascular

11. Gerasimovskaya E, Li M, Riddle SR, Frid MG. The adventitia: essen-

12. Archer SL, Wilkins MR. Basic science of pulmonary arterial
hypertension for clinicians: new concepts and experimental

13. Archer SL, Wilkins MR. Basic science of pulmonary arterial
hypertension for clinicians: new concepts and experimental

14. Archer SL, Wilkins MR. Basic science of pulmonary arterial
hypertension for clinicians: new concepts and experimental

15. Archer SL, Wilkins MR. Basic science of pulmonary arterial
hypertension for clinicians: new concepts and experimental

16. Archer SL, Wilkins MR. Basic science of pulmonary arterial
hypertension for clinicians: new concepts and experimental

17. Archer SL, Wilkins MR. Basic science of pulmonary arterial
hypertension for clinicians: new concepts and experimental
LTB4 activates adventitial fibroblasts

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 hypertension, acting on pulmonary artery endothelial, smooth muscle cells, and adventitial fibroblasts.

Novelty and Significance

- Activated macrophages, which are actively secreting LTB4, are concentrated in the outer adventitial layer of affected arterioles 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.
Leukotriene B4 Activates Pulmonary Artery Adventitial Fibroblasts in Pulmonary Hypertension


Hypertension. 2015;66:1227-1239; originally published online October 5, 2015; doi: 10.1161/HYPERTENSIONAHA.115.06370

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/66/6/1227

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2015/10/05/HYPERTENSIONAHA.115.06370.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
LTB₄ activates pulmonary artery adventitial fibroblasts in pulmonary hypertension.

Authors: Jin Qian¹,²,†, Wen Tian¹,²,†, Xinguo Jiang¹,², Rasa Tamosiuniene¹,², Yon K. Sung¹,², Eric M. Shuffle ¹,², Allen B. Tu¹,², Antonia Valenzuela², Shirley Jiang², Roham T. Zamanian², David F. Fiorentino², Norbert F. Voelkel³, Marc Peters-Golden⁴, Kurt R. Stenmark⁵, Lorinda Chung², Marlene Rabinovitch², Mark R. Nicolls¹,²,*

Affiliations:
¹ VA Palo Alto Health Care System, Palo Alto, CA 94304
² Stanford University, School of Medicine, Stanford, CA 94305
³ Virginia Commonwealth University, Richmond, VA 23284
⁴ University of Michigan Health Systems, Ann Arbor, MI 48109
⁵ University of Colorado Denver, School of Medicine, Aurora, CO 80045

† Contributed equally to manuscript
* Corresponding Author:
Mark R. Nicolls, MD
VA Palo Alto Health Care System; Stanford University School of Medicine
Med111P
3801 Miranda Ave.
Palo Alto, CA 94304
Office: (650) 493-5000 x69289
Fax: (650) 849-0553
mnicolls@stanford.edu
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.