Key Role of 15-Lipoxygenase/15-Hydroxyeicosatetraenoic Acid in Pulmonary Vascular Remodeling and Vascular Angiogenesis Associated With Hypoxic Pulmonary Hypertension

Cui Ma, Yaqian Li, Jun Ma, Yun Liu, Qian Li, Shengpan Niu, Zhiying Shen, Lei Zhang, Zhenwei Pan, Daling Zhu

Abstract—We have found that 15-hydroxyeicosatetraenoic acid (15-HETE) induced by hypoxia was an important mediator in the regulation of hypoxic pulmonary hypertension, including the pulmonary vasoconstriction and remodeling. However, the underlying mechanisms of the remodeling induced by 15-HETE are poorly understood. In this study, we performed immunohistochemistry, pulmonary artery endothelial cells migration and tube formation, pulmonary artery smooth muscle cells bromodeoxyuridine incorporation, and cell cycle analysis to determine the role of 15-HETE in hypoxia-induced pulmonary vascular remodeling. We found that hypoxia induced pulmonary vascular medial hypertrophy and intimal endothelial cells migration and angiogenesis, which were mediated by 15-HETE. Moreover, 15-HETE regulated the cell cycle progression and made more smooth muscle cells from the G0/G1 phase to the G2/M+S phase and enhanced the microtubule formation in cell nucleus. In addition, we found that the Rho-kinase pathway was involved in 15-HETE-induced endothelial cells tube formation and migration and smooth muscle cell proliferation. Together, these results show that 15-HETE mediates hypoxia-induced pulmonary vascular remodeling and stimulates angiogenesis via the Rho-kinase pathway. (Hypertension. 2011;58:679-688.) ● Online Data Supplement

Key Words: pulmonary hypertension □ pulmonary vascular remodeling □ angiogenesis □ 15-hydroxyeicosatetraenoic acid □ ROCK

Pulmonary hypertension (PH) is a refractory disease commonly associated with the high morbidity and mortality of adult and pediatric patients with various lung and heart diseases. The mechanisms by which the pulmonary arteries in the pulmonary circulation narrow include pulmonary vasoconstriction, pulmonary vascular remodeling, and thrombosis in situ. Pulmonary remodeling involves all 3 layers of the vascular wall and is complicated by the finding that cellular heterogeneity exists within the compartment of the pulmonary arterial wall. However, the specific role and the interaction between each cell are poorly understood. Generally, pulmonary vascular remodeling includes endothelial angiogenesis, smooth muscle cell proliferation and hypertrophy, adventitial fibroblast proliferation, myofibroblast differentiation, and extracellular matrix deposition. Hypoxia is considered as the predominant factor in the pathogenesis of pulmonary hypertension (PH). During the early period of hypoxic exposure, angiogenesis in the mature pulmonary circulation is a potentially beneficial adaptation for gas exchange. The lung vascular homeostasis involves maintaining an ideal number of capillaries per unit of lung volume. However, the sustained chronic hypoxia leads to disorder of the process and excess angiogenesis, which would impose more pressure on the proximal pulmonary artery and complicate the course of PH, suggesting that excess angiogenesis is a crucial player in the pathogenesis of PH.

Rho-kinase (ROCK), a downstream target of the GTPase RhoA, has been involved in many pathological processes, including endothelial dysfunction, vasoconstriction, and inflammation. It has been shown that ROCK regulates smooth muscle cell contraction and vascular tone in pulmonary circulations. The importance of ROCK in chronic hypoxic PH is highlighted because of its potential effects causing sustained vasoconstriction and the vascular wall cell proliferation and differentiation. However, the function of ROCK during angiogenesis and vascular remodeling in PH is controversial.

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In previous studies, we have found that hypoxia increases the formation of endogenous 15-hydroxyeicosatetraenoic acid (15-HETE) through stimulation of 15-lipoxygenase (15-LO). 13 15-HETE inhibits pulmonary artery smooth muscle cell (PASMC) apoptosis, 14 contributing to pulmonary vascular medial thickening. 15 These results raise the possibility of 15-HETE contributing to hypoxia-induced pulmonary vascular remodeling. Because both endogenous and exogenous 15-HETE increased the mRNA and protein expression of ROCK in PASMCs, 15 we hypothesize that 15-HETE mediates hypoxia-induced pulmonary vascular remodeling and angiogenesis through activation of the ROCK pathway. We observed that 15-HETE induced proliferation of PASMC and pulmonary artery endothelial cell (PAEC) migration and organization into capillary-like tube formation and increased the number of vascular vessels in chick chorioallantoic membrane assay, whereas Y-27632, the inhibitor of ROCK, markedly reversed the effect of 15-HETE.

Materials and Methods
For detailed Material and Methods, please see the online Data Supplement at http://hyper.ahajournals.org.

Animals and Lung Tissue Preparation
Adult male Wistar rats with a mean weight of 200 g were from the Harbin Medical University Experimental Animal Center, which is fully accredited by the Institutional Animal Care and Use Committee. Rats were randomly assigned to 9 days of normal and hypoxic environments with fractional inspired oxygen at 0.21 and 0.12, respectively, as described previously. 11 At the end of the 9-day exposure period, we anesthetized each rat and quickly removed the lungs.

Human lung specimens were obtained from lung transplantation surgery patients and were processed for histological studies. The presence of pulmonary vascular remodeling including the deposition of collagen and medial thickening was also found in medium-size pulmonary arteries obtained from rats exposed to hypoxia for 9 days. There was significant increase in wall thickness (Figure 1A) and in the deposition of collagen (Figure 1B) and medial thickening (Figure 1C) compared with normoxic rats. However, the increase was inhibited by administration of nordihydroguaiaretic acid (NDGA), a 15-LO inhibitor.

More importantly, we observed the expression of 15-LO-1/2 in human pulmonary vessels (Figure 2A, a, a1, c, and c1). Furthermore, 15-LO isozyme expression was upregulated in human PH, as shown by the intense staining with major upregulation of 15-LO-2 (Figure 2A, b, b1, d, and d1). The expression of 15-LO-1/2 was upregulated by hypoxia, as shown by the intense staining from hypoxic rats (Figure 2C, b, b1, e, and e1). However, when administered with NDGA, the increased expression was reversed (Figure 2C, c, c1, f, and f1).

Furthermore, we found that the endogenous 15-HETE was increased in rat lung tissues under hypoxic condition, which could be reduced by administration of NDGA (Figure S1A through S1C, available in the online Data Supplement). In addition, we identified 15-HETE with positive ion mass spectrum of the reaction product, 15-HETE-Derivatived, using an electrospray ionization liquid chromatography ion trap mass spectrometry; the peak at 566.3 was the derivatized 15-HETE plus a sodium ion (Figure S1D). These results suggest that the 15-LO/15-HETE pathway may be involved in the process of hypoxia-induced pulmonary vascular remodeling.

15-HETE Stimulated Bovine PAEC Migration and Tube Formation In Vitro and Chorioallantoic Membrane Angiogenesis In Vivo
To determine whether 15-HETE, the 15-LO metabolite of arachidonic acid, is angiogenic, we studied its effect on chick chorioallantoic membrane. We found that 15-HETE increased vessel density in chick chorioallantoic membrane compared with the control group in normoxia (Figure S2A). We further performed PAEC tube formation assay. Our results showed that exogenous 15-HETE stimulated PAEC tube formation compared with control in normoxia (Figure S2B). Our results also showed that the tube formation was increased by endogenous 15-HETE generated in hypoxia (Figure 3A). In the scratch-wound assay, the cells cultured in 5% FBS were exposed to hypoxia at 0, 6, 12, and 24 hours, respectively. We found that there were no significant changes in the migration within 6 hours of hypoxia exposure. After 24 hours, hypoxia induced significant migration compared with normoxic control (Figure 3B). Meanwhile, cinnamyl-3,4-dihydroxy-á-cyanocinnamate (CDC), the inhibitor of 15-LO, blocked the effect of endogenous 15-HETE on PAEC tube formation and migration. However, the inhibitory effect was partly diminished in the presence of exogenous 15-HETE (Figure 3A and 3B). These results indicated that 15-HETE is...
Figure 1. Hematoxylin-eosin staining (H&E), Masson staining, and the expression of $\alpha$-smooth muscle ($\alpha$-SM)–actin in pulmonary vessels from humans and rats. A. H&E staining of human lungs showed that the wall thickness in human pulmonary hypertension (PH) was higher compared with normal humans. B. The deposition of collagen in human lungs was increased in patients with PH. C. The $\alpha$-SM–actin expression was also increased in the pulmonary vascular wall in PH ($n=3$). In the animal experiment, hypoxia significantly increased the pulmonary vascular thickness (A), the deposition of collagen (B), and the expression of $\alpha$-SM–actin (C) compared with normoxic rats, which were reversible by administration of nordihydroguaiaretic acid (NDGA) (15-lipoxygenase inhibitor) to rats ($n=3$). Scale bars are 50 $\mu$m. Data show quantitative analyses of positive staining per vascular area (adventitia + media + intima + lumen). All of the values are denoted as means $\pm$ SEM; *$P<0.05$ vs normoxia group; #$P<0.05$ vs hypoxia group. Nor indicates normoxia; Hyp, hypoxia; N, NDGA.
a potent arachidonic acid–derived lipid mediator involved in the pulmonary vascular angiogenesis.

15-HETE–Mediated Hypoxia Induced PASMC Proliferation

To demonstrate the effect of 15-HETE on the PASMC proliferation, cell viability was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. We found that cell viability was increased by 15-HETE but not affected by CDC and NDGA (15-LO inhibitors) treatment on normoxic condition (Figure S3A). The column in Figure S3B show that hypoxia, known to promote the formation of endogenous 15-HETE, led to a significant increase in cell viability compared with normoxic condition. CDC and NDGA inhibited the effect in hypoxia. However, the decrease was reversed by adding the exogenous 15-HETE (Figure 4). We also analyzed the expression of the proliferating cell nuclear antigen (PCNA) in PASMCs. We found that hypoxia increased the expression of PCNA, and the effect was decreased in the presence of CDC; however, exogenous 15-HETE had a protective role in cell proliferation (Figure S3C).

Effect of 15-HETE on Cell Cycle Progression and Microtubule Dynamic Stability

To understand whether hypoxia affected the cell cycle progression through the 15-LO/15-HETE pathway, the number of cells in the different cell cycle phases was detected by flow cytometry, and the organization of microtubules in the mitosis was examined by immunofluorescence staining with α-tubulin. The results showed that endogenous 15-HETE increased the percentage of cells in the G2/M+S phase. CDC suppressed the cell cycle progression and made more PASMCs arrested at the G0/G1 phase. In contrast, the accumulation of the G2/M+S phase was increased in presence of exogenous 15-HETE (Figure 5A). As shown in Figure 5B, hypoxia enhanced the tubulin polymerization in the cell
nucleus compared with that in normoxia, whereas the microtubule formation was suppressed after treating the cells with CDC. Increased exogenous 15-HETE was accompanied by an increase in polymerized tubulin. Because cyclin A plays an important role in both the S and G2/M phases,17 we analyzed the expression of cyclin A in PASMCs. A significant increase in the expression of cyclin A was observed in the cells incubated with 15-HETE, but the effect was eliminated by CDC (Figure S3D). These results suggested that 15-HETE has an impact on the cell cycle activity and induces PASMC proliferation, eventually contributing to pulmonary vascular medial thickening.

15-HETE–Induced PAECs Migration, Tube Formation In Vitro, and Chick Chorioallantoic Membrane Angiogenesis In Vivo Required Activation of ROCK Pathway

The expression of ROCK was examined in PAECs. Our results showed that 15-HETE increased the ROCK II expression under normoxic condition (Figure S4A). Moreover, hypoxia upregulated the expression of ROCK II, which was inhibited by CDC. The expression was higher at the presence of exogenous 15-HETE (Figure S4B). To elucidate whether the ROCK pathway is involved in 15-HETE–induced angiogenic events, we blocked the ROCK pathway with Y-27632. The chick chorioallantoic membrane assay showed that Y-27632 reduced the capillary distension compared with eggs treated with 15-HETE (Figure S4C). In the scratch-wound assay, the group that blocked ROCK pathway with Y-27632 produced a significant inhibition of the migratory capability
of PAECs induced by endogenous 15-HETE. Moreover, after blocking the ROCK pathway, the migration in response to exogenous 15-HETE was also significantly decreased (Figure 6B). Consistently, PAEC tube formations induced by both endogenous and exogenous 15-HETE were reversed by a ROCK inhibitor (Figure 6A).

**ROCK Pathway Mediated the PASMC Proliferation Induced by 15-HETE**

Previous studies have suggested that 15-HETE increased the ROCK II activity after 24 hours of stimulation in PASMCs. It may be possible that 15-HETE–induced PASMC proliferation requires activation of the ROCK pathway. Figure 7

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**Figure 5.** A, 15-Hydroxyeicosatetraenoic acid (15-HETE) promoted pulmonary artery smooth muscle cell (PASMC) cycle progression and increased the percentage of cells in the S + G2/M phase. Hypoxia increased the cell number in the S plus G2/M phases compared with normoxia, which was reversible by cinnamyl-3,4-dihydroxy-α-cyano-cinnamate (CDC; 5 μmol/L) through inhibition of the generation of endogenous 15-HETE (n=3). B, Cells were fixed and stained with anti-α-tubulin to stain microtubules and 4',6-diamidino-2-phenylindole to stain chromosomes and chromatin. 15-HETE activated tubulin polymerization in the nucleus. The effect of hypoxia on polymerization was blocked by CDC (n=3). Scale bars are 20 μm. Data are presented as means±SEM; *P<0.05; **P<0.01. Nor indicates normoxia; Hyp, hypoxia; C, CDC; 15, 15-HETE.
demonstrated that the increased effect of endogenous 15-HETE on cell 5-bromodeoxyuridine incorporation was significantly suppressed by Y-27632, whereas application of exogenous 15-HETE had no detectable effect on DNA synthesis. The expression of PCNA induced by 15-HETE was also abrogated by the ROCK inhibitor (Figure S5A). Because Y-27632 is a chemical blockage of ROCK, we used RNA interference technology to knock down the expression of ROCK II. To assess the efficiency and specificity of RNA interference, intracellular expression of ROCK II was measured. Our results showed that the expression of ROCK II treated with non-targeted control siRNA was not different from expression in untreated control cells or cells treated with the transfection reagent alone (Figure S5B). Then the expression of PCNA was tested. Our results showed that the expression of PCNA was decreased after the ROCK II silencing, and application of exogenous 15-HETE had no detectable effect on PCNA expression (Figure S5C). These data suggested that the effect of 15-HETE on PASMC proliferation was mediated by the ROCK pathway.

15-HETE Improved Cell Cycle Progression and the Mitotic Spindle Formation via the ROCK Signaling Pathway in PASMCs

To elucidate whether the ROCK pathway participates in 15-HETE–mediated cell cycle activity, we blocked ROCK with Y-27632. In hypoxia, endogenous 15-HETE significantly forced more cells to enter the S and G2/M phases from the G0/G1 phase compared with normoxic cells. However, application of exogenous 15-HETE had no detectable effect on cell cycle activity after blocking the ROCK pathway with...
Y-27632 (Figure 8A). We also found that the effects of exogenous or endogenous 15-HETE–induced microtubule formation were reversed by the ROCK inhibitor (Figure 8B). To understand the role of ROCK in cell cycle progression, the expression of cyclin A was studied in PASMCs. We found that the effects of endogenous 15-HETE were reversed by Y-27632; the protective effect of exogenous 15-HETE was also weakened after blocking the ROCK pathway with Y-27632 (Figure S5D). Moreover, after treating PASMCs with small interfering RNA against ROCK II, 15-HETE–induced expression of cyclin A was inhibited (Figure S5E).

**Discussion**

Lipoxygenases form a heterogeneous family of lipid-peroxidizing enzymes that are classified as 5-, 8-, 12-, and 15-lipoxygenases. Both 15-LO-1 and 15-LO-2 are ex-
pressed in humans in a tissue-specific manner and convert arachidonic acid to 15-HETE.19,20 Identifying the function of 15-LO/15-HETE provides opportunities to elucidate the pulmonary vascular remodeling mechanisms and its role in PH development. A major finding of the study is that both 15-LO-1 and 15-LO-2 are overexpressed in the pulmonary vessels of human PH lungs. Moreover, NDGA, the 15-LO inhibitor, reverses the increases of media hypertrophy and extracellular matrix production induced by chronic hypoxic exposure in rats. In short, the evidence support the hypothesis that 15-LO/15-HETE is a major contributor of pulmonary vascular remodeling in PH.

It has been reported that angiogenesis constitutes a fundamental process underlying diverse physiological and pathological situations.21 However, little is known about the molecular mechanisms of the angiogenesis. Many factors, such as fibroblast growth factor, vascular endothelial growth factor, and platelet-derived growth factor, that influence endothelial cell migration and proliferation are likely responsible for the progression of angiogenesis in PH.22,23 In our study, hypoxic exposure induced the expression of 15-LO, which catalyzed the formation of 15-HETE. 15-HETE increases the number of new blood vessels in vivo and induces PAEC tube formation and migration in vitro. Thus, our study provides a new piece of evidence that hypoxic angiogenesis of pulmonary circulation is mediated through the 15-LO/15-HETE pathway.

Previous studies have shown that hypoxia stimulates PASMC proliferation, which is a key component of pulmonary vascular remodeling.24 There are reports that arachidonic acid metabolites stimulate growth and migration in vascular smooth muscle cells.25,26 Consistent with this notion, we have found that hypoxia-enhanced 15-HETE upregulates the proportion of cells in the S and G2/M phases and promotes polymerization of mammalian tubulin into microtubules in the mitosis phase. The process may be through direct or indirect stimulation of cyclins. All of the information presented here indicated that 15-HETE activates cell cycle progression in the process of PASMC proliferation.

Our previous reports have indicated that the ROCK pathway is involved in 15-HETE–induced pulmonary vasoconstriction in hypoxic rats.27 Accumulating evidence suggests that the RhoA/Rho kinase plays an important role in many pathophysiologic events, including mediation of sustained abnormal vasoconstriction, promotion of vascular inflammation, and remodeling.28 Our findings demonstrate that 15-HETE activates the ROCK signaling pathway in PAECs, and this activation of ROCK facilitates angiogenesis by increasing the PAEC migration. We also find that blockade of the ROCK pathway in PASMCs inhibits the expression of cyclin A, which is initially described as a “mitotic” cyclin acting. In addition, ROCK suppression decreases the number of cells in the S and G2/M phases in PASMCs. It is likely that ROCK activation is involved in the regulation of the activity of cell cycle regulatory proteins and cell cycle progression induced by hypoxia. Thus, our data provide a new molecular mechanism that 15-HETE–induced cell cycle progression is mediated by the ROCK pathway. However, whether any of the other downstream effectors of RhoA are involved in the process is still unknown.

In conclusion, our data show that the expression of 15-LO is apparently increased in human PH, and 15-HETE contributes to neointima formation and media hypertrophy induced by hypoxia through the ROCK signal pathway. In this regard, the regulation of the ROCK pathway by 15-HETE may be a significant contributor to PH. These findings appeal in favor of the potential relevance of the ROCK pathway and 15-HETE inhibition in the treatment of human PH.

Perspectives

Previous studies from our laboratory have shown that 15-HETE is an important mediator in hypoxic pulmonary arterial hypertension. Although we have proven that 15-HETE induces vasoconstriction and inhibits apoptosis in pulmonary artery smooth muscle cells, whether 15-HETE is attributable to the hypoxia-induced pulmonary vascular remodeling and angiogenesis needs to be studied further. Here, this study indicates that both 15-LO-1 and 15-LO-2 are overexpressed in the vessels of human PH. Moreover, 15-HETE stimulates tube formation and migration of PAECs and regulates the cell cycle progression resulting in pulmonary PASMC proliferation via the ROCK pathway. In view of these observations, it gives a clue in identifying the role of the 15-LO/15-HETE pathway in hypoxic angiogenesis of pulmonary circulation and its effect on cell cycle progression in the process of PASMC proliferation. Furthermore, we reveal a novel regulatory pathway that the regulation of ROCK pathway by 15-HETE may be an important mechanism underlying the treatment of PH and may provide a novel therapeutic insight. Future experiments need to be developed to discover specific ROCK inhibitors or inhibitors of 15-LO to ultimately design specific therapies in the clinic to turn the process off.

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Disclosures

None.

References


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Key Role of 15-LO/15-HETE in Pulmonary Vascular Remodeling and Vascular Angiogenesis Associated with Hypoxic Pulmonary Hypertension

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Running title: 15-LO/15-HETE in hypoxic pulmonary hypertension
Supplemental Materials
15-HETE, cinnamyl 3, 4-dihydroxy-[alpha]-cyanocinnamate (CDC), nordihydroguaiaretic acid (NDGA), Y-27632, 15-LO-1 and 15-LO-2 polyclonal antibodies were purchased from Cayman Chemical Company (Ann Arbor, USA). Growth factor-reduced Matrigel and the CycleTEST™ PLUS DNA Reagent Kit were obtained from BD Biosciences (Bedford, MA). Bromodeoxyuridine (BrdU) proliferation assay kit was purchased from Millipore Corporation (Billerica, MA). Antibodies against PCNA, Cyclin A and α-tubulin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). All other reagents were from common commercial sources.

Supplemental Methods
Animals and lung tissues preparation
Adult male Wistar rats with a mean weight of 200 g were from the Experimental Animal Center of Harbin Medical University, which is fully accredited by the Institutional Animal Care and Use Committee (IACUC). Adult male Wistar rats were randomized to 9 days of normal and hypoxic environments with fractional inspired oxygen (FiO2) 0.21 and 0.12, respectively as previously described 1. Normoxic rats were kept in the same room adjacent to the hypoxic chamber. To test the effects of nordihydroguaiaretic acid (NDGA) on hypoxia, one group of rats had been given NDGA (650 mg/kg b.w. orally, once daily) since 2 days before hypoxia until they were euthanized (the 10th day after hypoxia). At the end of the 9 days exposure period, we anesthetized each rat with pentobarbital injection (120 mg/kg, i.p.), opened the thorax and the lungs were quickly removed and further processed for immunocytochemistry as described below. Human lung specimens were obtained from lung transplantation (PH) or from lobectomy or pneumonectomy for localized lung cancer (Normoxia) in the Second Affiliated Hospital of Harbin Medical University under the procedures approved by the Ethnic Committee for Use of Human Samples of Harbin Medical University, then paraffin embedded, as described below.

Cell culture
Calf lungs, from local slaughterhouse, used in the study were approved by the Ethical Committee of Laboratory Animals at Harbin Medical University. Primary cultured PAECs were prepared from pulmonary arteries isolated from calf lungs. The arteries were slit open along their lengths and gently scratched along the intimal surface with a surgical blade. The purity and identity of PAECs were confirmed by positive immunofluorescence staining using antibodies to CD31 (Santa Cruz). PASMCs were collected as previously published protocol 2. Briefly, the vessels which were endothelial removed were dissected free of fat and excess adventitial tissue. Small fragments (about 1 cm²) were transferred to a flask. After adhered for 30 min, the arteries were covered with media Dulbecco's modified eagle's medium (DMEM) containing 20% fetal bovine serum (FBS). After 5 days, the tissue pieces were then lifted
out of the medium, and adherent smooth muscle cells were allowed to proliferate. The purity and identity of PASMCs were verified by immunocytochemistry staining using antibodies against smooth muscle α-actin.

**Morphometric Analysis**

The lung tissues were sliced into tissue blocks, and immersed in 4% paraformaldehyde for overnight fixation. Then fixed tissues were dehydrated, cleared, and embedded in paraffin wax. The paraffin blocks were cut into 5 µm thick sections. Some sections were stained with hematoxylin and eosin (H&E) and the others were stained with Masson trichrome. For immunohistochemistry, 5-µm paraffin-embedded tissue sections were deparaffinized and rehydrated in graduated alcohol. Then the tissue sections were treated in a 0.1 mol/L of sodium citrate buffer and heated for 20 min for antigen retrieval. After they were cooled down, the endogenous peroxidase activity was blocked, and then the sections were incubated with anti-α-SM-actin, anti-15-LO-1, anti-15-LO-2 antibodies (each concentration is 1:400, 1:50 and 1:200). Parallel controls were run with PBS. After an overnight incubation, the sections were washed three times with PBS and then subjected to the secondary antibodies (1:200) for the IgGs of the appropriate species. Following, sections were visualized with 3,3-diaminobenzidine (DAB) and counterstained using hematoxylin. Brown and yellow colors indicated positive stains. The total area of collagen and the positive staining area of α-SM-actin, 15-LO-1 and 15-LO-2 immunoreactivity in the vascular walls were quantified on high-resolution images of individual vessel by image analysis using a color-recognition algorithm in the soft of Image Pro Plus 6.0.

**MTT assay**

PASMCs were cultured in a 96-well culture plate (about $1 \times 10^4$), and then the cells were treated with CDC (5 μmol/L), NDGA (30 μmol/L), 15-HETE (1 μmol/L), 15-HETE plus CDC or 15-HETE plus NDGA or ethanol (vehicle) in DMEM with 5% FBS. Then the samples were exposed to hypoxia (3% O₂). Ethanol and other agents at the indicated concentrations were added every 24 h. At the end of the incubation in 37 °C, the cells were incubated for 4 h in a medium containing 0.5% 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT), the yellow mitochondrial dye. The amount of blue formazan dye formed from MTT is proportional to the number of survival cells. The reaction was terminated by adding DMSO to the medium followed by incubation for 10 min. The spectrophotometer absorbance at 540 nm was measured.

**Bromodeoxyuridine incorporation**

PASMCs were plated at $1\times10^5$ cells/well in 96-well plates, and then subjected to growth arrest for 24 h before exposed to hypoxia or treated with different
agents in 5% FBS DMEM. We measured BrdU incorporation using BrdU proliferation assay kits according to the manufacturer's protocol. Briefly, the cells were labeled with 10 ng/ml of BrdU during the incubation, washed 3 times with cold Wash Buffer, fixed, air-dried and incubated 1 h at room temperature with mouse anti-BrdU monoclonal antibody (diluted 1:200). The antibody was aspirated. The cells were washed 3 times and then incubated with peroxidase goat anti-mouse IgG (1:2,000) at room temperature for 30 min. The cells were washed 3 times, and 100 μl substrates were added to each well and incubated for 30 min in darkness. Thereafter, measured absorbance at dual-wave lengths of 450 to 540 nm was determined.

**Cell cycle and DNA analysis**
The CycleTEST PLUS DNA Reagent Kit was used for examining whether the cell cycle was influenced by hypoxia or 15-HETE. PASMCs were treated with Y-27632 (1 µmol/L) as previously described 3, CDC (5 µmol/L), 15-HETE (1 µmol/L), 15-HETE plus CDC or 15-HETE plus Y-27632 or ethanol (vehicle). The cells were harvested with trypsin and fixed using 70% ethanol. The ethanol was removed and the cells were incubated in 200 µl PBS. The cells were stained with propidium iodide according to the kit protocol. DNA fluorescence was measured and flow cytometry proceeded using BD FACSCalibur Flow Cytometer (Bedford, MA). For each sample, 2×10⁴ events were accumulated in a histogram. The proportions of cells in the different phases of the cell cycle were calculated from each histogram.

**Immunofluorescence study and microscopic observation**
PASMCs were cultured on a poly-L-lysine-coated cover glass (15 mm diameter) and washed three times with PBS, followed by fixation with 4% paraformaldehyde at room temperature for 15 min. After permeabilization with 0.01% Triton X-100 for 10 min, the cells were blocked with 3% normal bovine serum at 37 °C for 30 min, followed by incubation with anti-α-tubulin primary antibodies (1:50) in PBS at 4 °C overnight. After washed three times with PBS, the cells were incubated with FITC-conjugated secondary antibody (1:100) diluted by PBS at 37 °C for 2 h and DAPI away from light. Then the cover glass was mounted and examined with confocal laser scanning microscope (CLSM). The images were merged by CLSM.

**CAM assay**
Fertilized chick eggs were incubated at 37 °C in the incubator with 60±10% relative humidity. After 7 days of incubation, a small hole was broke in the superior surface of the eggshell, and various test factors were applied on a 0.5% solution of methylcellulose in distilled water, 20 μl of which were allowed to dry at 60 °C and placed on the CAMs. After 24 h the chick chorioallantoic membranes (CAMs) were fixed by methanol and acetone and then placed onto a microscope slide. Images were recorded by digital photomicrography.
(Olympus, Japan). For each sample, a 0.1×0.1-cm grid was added to the digital CAM images and the average number of vessels within 5-7 grids was counted as a measure of vascular density.

**Tube formation assay**

Ninety-six-well culture plates (Costar, Corning) were coated with growth factor-reduced Matrigel (BD Biosciences) in a total volume of 30 µl and allowed to solidify for 30 minutes at 37 °C. PAECs were trypsnized and resuspended at 5×10⁴/mL and 200 µl of this cell suspension were added into each well. Vehicle or chemicals of interest at the indicated concentration were added to the appropriate well. Tube formation was observed under an inverted microscope (Nikon, Japan). Tube length was measured using Image Pro Plus 6.0.

**Scratch-Wound assay**

The confluent PAECs cultured in 6-well plates were wounded by pipette tips, given rise to one acellular 1-mm-wide lane per well, and the ablative cells were washed out by PBS. After that, cells were treated with vehicle or chemicals of interest with 5% FBS DMEM. Wounded areas were photographed at zero time. After 6 h, 24 h of incubation, photos were taken from the same areas as those recorded at zero time.

**siRNA Design and Transfections**

To silence the expression of ROCKII protein, PASMCs were transfected with small interfering RNA, which was designed and synthesized by GenePharma. Non-targeted control siRNA (siNC) was used as negative control. The sense sequence of siRNA against ROCKII and non-targeted control sequence were listed below: accession No.ds-siRNA sequence corresponding nucleotides, ROCKII: (NM_174452) 5’-GCCAUACACUCCAUGGGUUTT-3’, NC control: 5’-UUCUCCGAACGUGUCACGUTT-3’. Briefly, the PASMCs were cultured till 30%-50% confluence and then 2 µg siRNA and 10 µl X-tremeGene siRNA Transfection Reagent were respectively diluted in serum-free Opti-MEM-1 medium and mixed them together. Incubated the mixture (siRNA/Transfection Reagent) at room temperature for 20 min and added directly onto cells. After transfections, cells were quiesced for 48 h and used as required.

**Western blot Analysis**

Cultured cells were treated with CDC (5 µmol/L), Y-27632 (1 µmol/L), 15-HETE (1 µmol/L), 15-HETE plus CDC or 15-HETE plus Y-27632 or ethanol (vehicle) in DMEM with 5% FBS for 24 h. Proteins were solubilized and extracted with 300 µl lysis buffer (Tris 50 mM, pH 7.4, NaCl 150 mM, Triton X-100 1%, EDTA 1 mM, and PMSF 2 mM) and incubated for 30 min on ice. Then the lysates were sonicated and centrifuged at 12,000 rpm for 10 min, and the insoluble fractions were discarded. The protein concentrations in the supernatant were
determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. Equal amounts of protein (20 µg) from each sample were subjected to electrophoresis on an SDS-polyacrylamide gel, and transferred onto nitrocellulose membrane (Millipore, USA). After 1 h incubation in a blocking buffer (Tris 20 mM, pH 7.6, NaCl 150 mM, and Tween 20 0.1%) containing 5% nonfat dry milk powder, the membranes were reacted with appropriate antibody to PCNA, Cyclin A and ROCK II at 1:500 overnight at 4 °C. Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents.

**Reverse-Phase High-Pressure Liquid Chromatography (RP-HPLC) and Mass spectrometry**

The contents of 15-HETE in rat lung tissues which contain pulmonary arteries were analyzed by RP-HPLC according to the published method \(^1,4\). Briefly, the tissues were homogenized within ethyl acetate which was acidified to pH 3.0 with formic acid and centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatants were collected, dried down under nitrogen, reconstituted in 0.8 ml of 20% acetonitrile:water (pH 3.0), and applied to a Sep-Pak Vac that was pre-washed with water followed by acetonitrile and water. The column was washed with different proportional acetonitrile: water to remove polar lipids and then was eluted with 500 µl of ethyl acetate to capture the free fatty acids. The samples were labeled with 2-(2,3-naphthalimino)-ethyl trifluoromethanesulfonate (36.4 mmol/l). N,N-diisopropylethylamine was added to catalyze the reaction. Endogenous 15-HETE was separated on an ODS column (4.6 mm × 250 mm, 5 µm) at 1.3 ml/min isocratically with methanol/water/glacial acetic acid (80 : 20 : 0.1) and detected with fluorometer. Agilent 6310 ion channel mass spectrum was used to prove the derivatized 15-HETE. Mass spectrometry condition is as follows: Nebulizer:30.0 PSI; Dry gas:10.0 L/MIN; DRY TEMP:352°C; Capillary:3500 v. Positive ion mass spectrum of the reaction product, 15-HETE-Derivatived, using an electrospray ionization liquid chromatography ion trap mass spectrometry. Relative abundance of the ions is plotted against the mass-to-charge ratio (m/z).

**Statistical analysis**

The composite data are expressed as means ± SEM. Statistical analysis was performed with Student's t-test or one-way ANOVA followed by Dunnett's test where appropriate. P<0.05 was considered statistically significant.
Supplemental References
Supplemental Figures

Figure S1

Figure S1:
Measurement and identification of endogenous 15-HETE levels by RP-HPLC and mass spectrometry. (A-C) RP-HPLC showed that hypoxia induced the generation of endogenous 15-HETE in lung tissues, but administration of NDGA to rats decreased the level of endogenous 15-HETE (n = 3). Data are presented as means ± SEM. *P < 0.05. (D) Mass spectrometry analysis showed that the peak at 566.3 was the derivatized 15-HETE plus a sodium ion. “Nor” means Normoxia, “Hyp” means Hypoxia.
Figure S2:

15-HETE induced CAM angiogenesis *in vivo* and tube formation *in vitro* in normoxia. (A) Images depicted angiogenic response to 15-HETE in chorioallantoic membrane assay. Fewer allantoic small vessels were seen radially in the control group (n = 6). Scale bars are 100 µm. (B) The PAECs tube formation in normoxia. 15-HETE (1 µmol/L) significantly stimulated PAECs tube formation (n = 3). Scale bars are 50 µm. All values are denoted as mean ± SEM. *P < 0.05.
Figure S3:
15-HETE increased PASMCs proliferation. (A) The data indicated that cell viability was not changed on treatment with CDC or NDGA in normoxia, but 15-HETE (1 µmol/L) alone increased the cell viability in these cells (n = 6). Data are presented as means ± SEM. *P < 0.05 versus control group. (B) Both CDC and NDGA inhibited the protective effect of 15-HETE on cell viability in hypoxic condition (n = 6). (C) The increase of PCNA expression induced by hypoxia and exogenous 15-HETE was suppressed after treating the cells with CDC (n = 3). (D) Both endogenous and exogenous 15-HETE increased the expression of Cyclin A. CDC inhibited the effect of endogenous 15-HETE induced by hypoxia (n = 3). Data are presented as means ± SEM. *P < 0.05 versus normoxia group; #P < 0.05 versus hypoxia group.
Figure S4:
15-HETE increased the expression of ROCKII in PAECs and pharmacologic blockade of ROCKII suppressed CAM angiogenesis. (A) Under normoxic condition 15-HETE up-regulated the protein expression of ROCKII compared with control. (B) Under hypoxic condition, the expression of ROCKII was increased by 15-HETE, which was reversible by CDC (n = 3). (C) Y-27632 obviously decreased the number of allantoic capillaries induced by 15-HETE (n = 6). Scale bars are 100 µm. Data are presented as means ± SEM. *P < 0.05; **P < 0.01. “Y” means Y-27632.
Figure S5:
(A) The expression of PCNA under hypoxic condition was partly inhibited by Y-27632 while application of exogenous 15-HETE had no detectable effect on PCNA expression after blocking the ROCK pathway with Y-27632. (B) The efficiency and specificity of siRNA directed against ROCKII. (C) The expression of PCNA after the treatment of siRNA against ROCKII was decreased. (D) The effect of endogenous and exogenous 15-HETE on the Cyclin A expression in PASMCs was decreased after the treatment of Y-27632 (n = 3). (E) The effect of endogenous and exogenous 15-HETE on the Cyclin A expression in PASMCs was decreased after the treatment of siRNA against ROCKII. Values are denoted as mean ± SEM from at least three separate experiments. *P < 0.05; **P < 0.01.