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15-Lipoxygenase and 15-hydroxyeicosatetraenoic acid regulate intravascular thrombosis in pulmonary hypertension

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Shen T, Shi J, Wang N, Yu X, Zhang C, Li J, Wei L, Ma C, Zhao X, Lian M, Jiang C, Zhu D. 15-Lipoxygenase and 15-hydroxyeicosatetraenoic acid regulate intravascular thrombosis in pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 309: L449–L462, 2015. First published June 19, 2015; doi:10.1152/ajplung.00004.2015.—Pulmonary arterial hypertension (PAH) is a disease characterized by thickening of pulmonary artery walls, elevated pulmonary vascular resistance, pulmonary vascular thrombotic lesions, and right heart failure. Recent studies suggest that 15-lipoxygenase (15-LO)/15-hydroxyeicosatetraenoic acid (15-HETE) play an important role in PAH, acting on arterial walls. Here, we show evidence for the action of the 15-LO/15-HETE signaling in the pulmonary vascular thrombotic lesions in the experimental PAH models. Platelet deposition was augmented in rats exposed to hypoxia and Sugen 5416, which were both prevented by nordihydroguaiaretic acid (NDGA), a 15-LO inhibitor. Chronic hypoxic resulted in the platelet deposition specifically in pulmonary vasculature, which was reversed by 15-LO inhibitor. The 15-LO pathway mediated in the endothelial dysfunction induced by hypoxia in vivo. Meanwhile, 15-HETE positively regulated the generation of IL-6 and monocyte chemoattractant protein-1 (MCP-1). The coagulation and platelet activation induced by hypoxia were reversed by 15-LO inhibitor NDGA or the MCP-1 inhibitor synthesis inhibitor bindarit in rats. The 15-LO/15-HETE signaling promoted the coagulation and platelet activation, which was suppressed by MCP-1 inhibition. These results therefore suggest that 15-LO/15-HETE signaling plays a role in platelet activation and pulmonary vascular thrombosis in PAH, involving MCP-1.

thrombosis; 15-lipoxygenase; monocyte chemoattractant protein-1; platelet

PULMONARY ARTERIAL HYPERTENSION (PAH) is a devastating disease characterized by elevated pulmonary circulation resistance resulting in right heart failure and death (48). Pulmonary vasoconstriction, endothelial dysfunction, and vascular smooth muscle cell proliferation are likely attributable factors to PAH.

Another important event is thrombosis seen in 20–56% of patients with sporadic or familial PAH (47). Widespread thrombosis occurs in the pulmonary microvasculature contributing to the increase in pulmonary vascular resistance, cardiac overload and right ventricular stretch in pathogenesis of fatal PAH (3, 17). Several risk factors for thrombosis are seen in the pulmonary vasculatures such as endothelial dysfunction, inflammation, platelet activation and fibrinolysis as suggested by the increased plasma levels in von Willebrand factor (vWF), P-selectin, and plasminogen activator inhibitor type-1, and activated fibrinogen receptors GPIb/IIIa (5, 21, 37, 41, 46). However, it is unclear why the thrombosis takes place in pulmonary arteries (PA) but not in systemic vasculatures.

Unlike the blood in the systemic circulation, the O2 content in pulmonary arterial blood is very low, where the PO2 level is normally ~10 mmHg, compared with 40–50 mmHg in arterioles of the systemic circulation (9). During hypoxia, the PO2 levels in both circulations drop sharply, making the pulmonary arteries nearly anoxic. Such an extremely hypoxic condition has a profound impact on cells in the pulmonary arterial wall. Because of the lack of O2 in the lumen of PAs, cells rely heavily on O2 supplies from the interstitial space. O2 may diffuse through the vessel wall in a sequence, with the endothelium last. Therefore, the pulmonary arterial endothelium tends to be subjected to the lowest PO2 level in the entire circulation system, making these cells highly vulnerable to hypoxic injuries.

The hypoxic vascular endothelial injuries may be worsened by proinflammatory mediators, raising the tendency of blood coagulation in pulmonary circulation, because they may lead to the formation of microthrombi, an increase in arterial resistance, and the elevation in PA pressure. To test this hypothesis, we performed these studies. We found that the 15-lipoxygenase (15-LO) pathway regulated the endothelium dysfunction, platelet activation, proinflammatory cytokine release, and intravascular thrombosis, contributing to the development and maintenance of PAH.

MATERIALS AND METHODS

Human blood samples. Venous blood samples were obtained with local ethics committee approval and informed, written consent from healthy volunteers and idiopathic PAH patients. Plasma samples were obtained from 21 consecutive patients in the Harbin Medical University Second Affiliated Hospital, between 2012 and 2014. The work was approved by the Harbin Medical University Ethical Committee for Use of Human Samples.
Animals and treatments. Adult male Wistar rats (average weight of 200 g) from the Harbin Medical University Experimental Animal Center were randomly assigned to groups of control rats, hypoxia rats, hypoxia with nordihydroguaiaretic acid (NDGA, Cayman, CAS 500-38-9) rats, or hypoxia with bindarit rats, as described previously (52). Hypoxia with NDGA rats or hypoxia with bindarit rats were defined as hypoxia rats treated with NDGA (650 mg/kg body wt, orally once daily, for 11 days) (2, 15) or MCP-1 synthesis inhibitor bindarit (Abcam, ab143292, 100 mg/kg body wt, orally once daily, for 11 days) (42) from 2 days before hypoxia till they were anesthetized. Adult male Wistar rats were exposed to hypoxia (10% O2) and the vascular endothelial growth factor receptor antagonist Sugen 5416 (su5416; Sigma), as previously described (23, 38). Lung samples were obtained after 3 wk after su5416/H11001 hypoxia exposure (SuHx). Rats upon SuHx administration were treated with NDGA (650 mg/kg body wt, orally once daily, for 14 days) from 7 days after SuHx administration till they were anesthetized. All experimental procedures in animals followed the guidelines of and were approved by the Institutional Animal Care and Use Committee; all surgery was performed under pentobarbital sodium anesthesia; and all efforts were made to minimize suffering.

Morphometric analysis of the pulmonary artery, cardiac artery, and muscular artery. The left lung tissues were sliced into tissue blocks and immersed in 4% paraformaldehyde for overnight fixation (27). 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 vWF (Santa, Sc-6602, 1:50) antibodies. Parallel controls were run with 0.1 mol/l phosphate buffer (pH 7.4, 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. The sections were visualized with 3,3-diaminobenzidine and counterstained with hematoxylin. The total area of collagen and the positive staining area of vWF (brown and yellow colors) in the vascular walls were quantified on high-resolution images of individual vessel by using a color-recognition algorithm in Image Pro Plus 6.0.

Meanwhile, the right lung tissues, the isolated aorta, the isolated common carotid artery, the isolated mesenteric artery, the whole heart, the whole liver, the whole spleen, and the segmental muscle of the thigh were fixed in 4% paraformaldehyde for 6 h as reported (39). All these tissues were frozen in Tissue-Tek OCT compound (Sakura Finetechnical) at −20°C. Then, 7-μm sections were cut by using a cryostat. The cryosections were blocked with 10% normal goat serum/PBS for 30 min. Integrin αIIb antibody (Santa, Sc-6602, 1:25) was incubated at 4°C overnight. After being washed three times with PBS, sections were incubated with Alexa Fluor 546-conjugated goat anti-mouse antibody (Invitrogen, A-11018) for 2

Fig. 1. Platelet activation induced by Sugen 5416 (su5416) + hypoxia exposure (SuHx) administration was inhibited by nordihydroguaiaretic acid (NDGA). CD41 expression was increased in arterioles (A) and in small pulmonary vessels (B) exposed to hypoxia and su5416 compare with control animals. Sections were examined with a NA1.4 inverted Leica DMi6000 microscope (Leica, Heidelberg, Germany), and images were visualized by Hamamatsu ORCA-R2 camera (Hamamatsu, Japan) and recorded by LAS AF software (Leica). Quantification of staining intensity was expressed as units of intensity per field of vision. Ctr, control; N, NDGA. **P < 0.05; n = 5 rats.
h at room temperature and incubated with DAPI (Boster, AR1176) for 15 min in dark. Sections were washed three times with PBS and then examined with a NA1.4 inverted Leica DMI6000 microscope (Leica, Heidelberg, Germany), and images were visualized by Hamamatsu ORCA-R2 camera (Hamamatsu, Japan) and recorded by LAS AF software (Leica).

**Vascular casting.** Casts of the pulmonary vasculature were generated by injecting 5 ml of Microfil (Flow Tech, Carver, MA) into the pulmonary artery. Lungs were cured at 4°C overnight. Tissues were cleared with SCALEA2 solution (Olympus, Center Valley, PA) for 1–2 wk. The right upper lungs were visualized by ImageScanner III (EPSON, Japan).

**Cell culture.** We isolated human peripheral blood mononuclear cells (PBMCs) from citrated venous blood after overnight fasting as previously reported (37a). Blood samples were centrifuged immediately at 500 g for 10 min at 4°C to obtain platelet-rich plasma.

![Fig. 2. Immunofluorescence for platelet-specific marker CD41, Microfil pulmonary vascular casting, and immunohistochemistry for von Willebrand factor (vWF).](http://www.ajplung.org/fig2.jpg)
removal of the plasma, the platelet-free pellet was suspended in RPMI 1640 (1:3, vol:vol) (ICN Biomedicals) supplemented with 2 mmol/l glutamine, 0.5% streptomycin-penicillin-Fungizone, and 10% fetal calf serum and centrifuged over Ficoll-Paque (Pharmacia, Fine Chemicals) gradient at 1,000 g for 20 min.

Mononuclear cells were collected from the interface of the Ficoll medium, suspended in RPMI 1640, and incubated in 35-mm plastic dishes for 90 min at 37°C in 5% CO₂ humid atmosphere. The monocytes were collected by their adherence to dishes, and the lymphocytes (nonadherent cells) were removed by aspiration with

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**Fig. 3.** Immunofluorescence for CD41 in the systemic arteries. The CD41 expression in the aorta (A), the common carotid artery (B), and the mesenteric artery (C) were similar between hypoxic and normoxic groups. There was no significant difference in the platelet deposition of the small arteries between hypoxic and normoxic groups in the systemic circulation including the heart artery (D), liver artery (E), kidney artery (F), spleen artery (G), and muscle artery (H). Framed areas in A1–H1 and A2–H2 are shown at high magnification in a1–h1 and a2–h2, respectively. Quantification of staining intensity was expressed as units of intensity per field of vision. Nor, normoxia; Hyp, hypoxia; NS, no significance; n = 5 rats.
Pasteur pipette and washing of the dishes with RPMI 1640. Cell preparations were 90% monocytes, as determined by nonspecific esterase staining. After isolation, cells were incubated at 1×10⁶ cells/ml in RPMI 1640 at 37°C, 5% CO₂ for 6 h. Cultures were incubated in presence or absence of siRNA plus 15-hydroxyeicosatetraenoic acid (15-HETE) (1 mM) in 5% FBS-RPMI 1640 the others were exposed to hypoxia in the absence or presence of the inhibitor of 15-LO, cinnamyl-3,4-dihydroxy-acyanocinnamate (CDC) (5 μM), NDGA (30 μM). The cells cultured in complete medium were used as control. CDC, NDGA, and 15-HETE at the indicated concentrations were replaced every 24 h with new medium.

siRNA design and transfections. To silence the expression of 15-LO-1/2 protein, PBMCs were transfected with small interfering RNA, which was designed and synthesized by GenePharma. Nontargeted control siRNA was used as negative control. The sense sequence of siRNA against 15-LO-1, 15-LO-2, and nontargeted control sequence are listed with accession numbers as follows: 15-LO-1 (NM_031010.2), 5′-CCUCUAGUCAUGCUGAAUUTT-3′; 15-LO-2 (NM_153301.2), 5′-GGAGUCUGAUGAGAUGAATT-3′; NC control, 5′-UUCUCCGAACGUGUCACGUTT-3′. Briefly, the PBMCs were cultured until 50–70% confluence and then 1.5 g siRNA and 7.5 l X-tremeGene siRNA Transfection Reagent were separately diluted in 100 l serum-free Opti-MEM-1 medium and were mixed together 5 min later. The mixture (siRNA/Transfection Reagent) was incubated at room temperature for 20 min and added directly onto the cells. Cells were quiesced for 24 h and used as required.

Western blot analysis. Plasma from rats (normoxia, hypoxia, and hypoxia with NDGA) were homogenized in ice-cold storage PBS. The homogenates were sonicated on ice and then centrifuged at 16,099 g for 10 min at 4°C. The supernatants were collected and stored at −80°C until used in Western blot analysis.

After treatments in six-well culture clusters for 24 h, the cells were lysed in a RIPA buffer containing phosphatase inhibitor and incubated for 30 min on ice. The lysates were then sonicated and centrifuged at 16,099 g for 10 min, and the insoluble fraction was discarded. The supernatants were collected and stored at −80°C until used in Western blot analysis.

Protein concentrations were determined by the Bradford assay using BSA as standard. Plasma containing 50 μg of protein and cells protein samples containing 20 μg of protein were separated by SDS-PAGE as previously described. After electrophoresis, proteins were transferred to nitrocellulose sheets. These membranes were blocked in 5% milk and incubated with 15-LO-1 (Abcam, Ab119774, 1:400), 15-LO-2 (Abcam, Ab111171, 1:400), MCP-1 (Boster, BA1255, 1:100), IL-6 (Boster, BA4339-2, 1:100), β-actin (Santa Cruz, sc-47778, 1:2,000), and secondary antibodies as described. These proteins were visualized with enhanced chemiluminescence reagents (SuperSignal, Pierce).

Procoagulant activity and inhibition assays of platelets. PCA of platelets was evaluated by one-stage recalcification time assay in a KC4A-coagulometer (Amelung, Labcon, Heppenheim, Germany), and 100 μl of platelet (1×10⁷) was incubated at 37°C. After 180 s,
100 µl of warmed 25 mM CaCl₂ was added to start the reaction and the clotting time was recorded. All clotting assays were performed in triplicate. For the inhibition assay of coagulation time, 50 µl ADP (final concentration 128 nM), anti-MCP-1 (final concentration 40 µg/ml), or 15-HETE (final concentration 1–20 μM) was incubated with 100 µl cell for 10 min at 37°C. Clotting time was then recorded as above after addition of 50 µl of warmed 50 mM CaCl₂ (12).

Measurement the level of 15-HETE. The 15(S)-HETE EIA kit (cat. no. 534721, Cayman) was utilized for the detection of the amount of 15(S)-HETE. Peripheral blood mononuclear cells were lysed in a 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 amount of endogenous 15-HETE was measured by 15(S)-HETE EIA kit. The protein concentrations were determined by Bradford protein assay. The results were analyzed with Cayman Chemical Company Enzyme Immunoassay (EIA) Tools.

ELISA. Plasma supernatants were obtained for platelet factor 4 (PF4) measurement by enzyme-linked immunosorbent assay (ELISA) (Human CXCL4/PF4 ELISA; R&D Systems, Minneapolis, MN). β-Thromboglobulin (β-TG) levels and fibrinogen were measured in citrate plasma by means of ELISA (R&D Systems). D-dimer levels were measured in citrate plasma by means of ELISA Zymutest D-dimer (Hyphen BioMed, Neuville-sur-Oise, France).

The concentration of IL-1β, IL-6, IL-8, MCP-1, TNF-α, and TGF-β in plasma were assessed in citrated plasma separately by use of Human IL-1β Quantikine, Human IL-6 Quantikine HS, Human CXCL8/IL-8 Quantikine ELISA kit, Human CCL2/MCP-1 Quantikine ELISA kit, Human TNF-α Quantikine ELISA kit, and Human TGF-β1 Quantikine ELISA kit (R&D Systems) by means of ELISA assay.

Ultraperformance liquid chromatography. The contents of 15-HETE in human and rat peripheral blood were analyzed by ultraper-

Fig. 5. 15-LO pathway mediated the platelet activation in the human plasma. Activated fibrinogen receptors (A) and platelet cell-surface P-selectin (B) after 15-HETE addition (5 μM) for 0.5 h, ADP (5 μM) for 0.5 h, and ADP with NDGA administration (30 μM) for 0.5 h were detected by FACSscan flow cytometer (Becton Dickinson). The concentrations of β-thromboglobulin (β-TG; C) and platelet factor 4 (PF4; D) in the plasma was determined by ELISA (BioTek). Ctr, control; siMCP1, silencing the gene of MCP1 with small interfering RNA; siIL-6, silencing the gene of IL-6 with small interfering RNA. *P < 0.05, n = 5 rats. All values are denoted as means ± SE.
formance liquid chromatography (UPLC) according to the published method (28, 52). Briefly, the tissues were homogenized within ethyl acetate, which was acidified to pH 3.0 with formic acid and centrifuged at 15,000 rpm for 20 min at 4°C. The supernatants were collected, dried down under nitrogen, reconstituted in 0.8 ml of 20% acetonitrile:distilled water (pH 3.0), and applied to a Strata-X polymeric SPE column that had been preconditioned with 5 ml of 100% ethyl alcohol and 5 ml of 25% ethyl alcohol followed by distilled water and 25% ethyl alcohol. Thereafter, the eicosanoids were eluted from the column with ethyl acetate containing 5 ml of 0.0002% butylated hydroxytoluene. Endogenous 15-HETE was separated on a Waters Acquity UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 µm) which was maintained at 30°C. The mobile phase consisted of 0.05% aqueous formic acid (B) and methanol/acetonitrile (1:4 vol/vol) (A) at a flow rate of 0.25 ml/min. The mobile phase gradient was ran from 30 to 100% A for 7 min, returned to 30% A for 1.5 min, and was held at 30% A for 1.5 min for reequilibration. A time-of-flight mass spectrometer equipped with an electrospray ionization interface (Waters, LCT premier XE) was used in detecting the negative ionization of 15-HETE.

Flow cytometry for detection of platelet activation. One 5-µl aliquot from each whole-blood or platelet-rich plasma sample was added to tubes containing saturating concentrations of either phycoerythrin-labeled PAC-1, representing activated fibrinogen (activated GPIIb/IIIa) receptors and allophycocyanin (APC)-labeled CD-61, or FITC-labeled CD-62P and APC-labeled CD-61. Samples were incu-

Table 1. Correlations between 15-HETE production with pulmonary hemodynamic, physiological, and biological variables in 21 patients with idiopathic pulmonary arterial hypertension disease in plasmid

<table>
<thead>
<tr>
<th>Characteristics</th>
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<tr>
<td>Age, yr</td>
<td>0.03</td>
<td>0.91</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>−0.02</td>
<td>0.94</td>
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<tr>
<td>Systolic Pap, mmHg</td>
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<td>0.52</td>
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<td>Pap, mmHg</td>
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<td>Pcw, mmHg</td>
<td>0.37</td>
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<td>MPAD, mm</td>
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<td>0.11</td>
</tr>
<tr>
<td>CI, l·min⁻¹·m⁻²</td>
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<td>0.83</td>
</tr>
<tr>
<td>PVR, units/mm²</td>
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<td>0.001</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
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<td>0.03</td>
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<tr>
<td>MCP-1, pg/ml</td>
<td>0.68</td>
<td>0.001</td>
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<tr>
<td>IL-1β, pg/ml</td>
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</tr>
<tr>
<td>IL-8, pg/ml</td>
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<td>0.23</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>0.16</td>
<td>0.49</td>
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<tr>
<td>TNF-α, pg/ml</td>
<td>−0.16</td>
<td>0.49</td>
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15-HETE, 15-hydroxyeicosatetraenoic acid; BMI, body mass index; Pap, mean pulmonary artery pressure; Pcw, pulmonary capillary wedge pressure; MPAD, main pulmonary artery diameter; PVR, pulmonary vascular resistance; CI, cardiac index; WU, wood units; IL, interleukin; MCP, monocyte chemoattractant protein-1; TGF, transforming growth factor; TNF, tumor necrosis factor. Numbers indicate the Spearman correlation coefficient (r) and corresponding P value.

Fig. 6. Neither MCP1 or IL-6 could regulate the 15-LO1/2 protein expression. The transfection efficiency of MCP-1 (A) and IL-6 (B) was shown. C: protein expression of 15-LO1/2 after MCP-1 siRNA addition. D: protein expression of 15-LO1/2 after IL-6 siRNA addition. Nor, normoxia; Hyp, hypoxia; NC, negative control. n = 5 rats. *P < 0.05, **P < 0.05, n = 5 rats. All values are denoted as means ± SE.
bated in the dark at room temperature for 15 min, fixed with 1 ml of cold 1% paraformaldehyde solution, and stored at 4°C. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson). Platelets were distinguished by the characteristic light scatter and the platelet-specific antibody CD61 binding to GPIIb/IIIa, the fibrinogen receptor. After appropriate color compensations were defined, the percentage of platelets positive for PAC-1, representing activated fibrinogen (activated GPIIb/IIIa) receptors, or CD62-P, representing the surface expression of P-selectin (a marker of platelet α-granule release), were calculated from 10,000 events positive for CD-61 with a fluorescent intensity. Pilot experiments showed no significant interference of by-anti-CD61 on PAC-1 binding to platelets (data not shown). For the purposes of comparison, platelet activation levels were also analyzed in the assays on patients in steady state by determining the mean fluorescence intensity of the PAC-1 or anti-CD62-P staining. We elected to present the platelet activation data in this paper as percentage above threshold, consistent with most other similar published studies.

Statistical analysis. The data are presented as means ± SE. Statistical analysis was performed with Student’s t-test or one-way ANOVA followed by a Dunnett’s test where appropriate. P < 0.05 was considered statistically significant.

RESULTS

Platelet deposition, pulmonary thrombosis, and endothelial dysfunction were regulated by 15-LO/15-HETE pathway. To determine whether 15-LO/15-HETE signaling affects the platelet deposition in the pulmonary vasculature, immunofluorescence of the platelet-specific antigen GPIIb/IIIa (CD41) was evaluated in lung tissue sections. SuHx animal models, which have been established as the model of severe PAH, are shown to develop obliterative vascular lesions that are similar to the plexiform lesions found in idiopathic PAH patients (1). SuHx administration resulted in plentiful deposition of platelets in small arteries (50–200 μm) and arterioles (<50 μm) in rats. In contrast, platelet adhesion in normoxic animals was scarcely seen. We observe that the platelet deposition was more obvious in SuHx rats than control rats in some small pulmonary vessels (Fig. 1, A and B). Inhibition of the exogenous 15-HETE production with NDGA restrained the platelet deposition in pulmonary vasculatures in the SuHx rats (Fig. 1, A and B).

Fig. 7. 15-LO regulated the protein expression of MCP-1. The transfection efficiency of 15-LO1 (A) and 15-LO2 (B) was shown; n = 5. Protein expression of MCP-1 (C) and IL-6 (D) after phytohemagglutinin (PHA, 5 μM), PHA (5 μM) with NDGA (30 μM), PHA (5 μM) with CDC (5 μM), PHA (5 μM) with 15-LO1 siRNA, and PHA (5 μM) with 15-LO2 siRNA addition. Ctr, control; Hyp, hypoxia. *P < 0.05, **P < 0.01 compared with control; #P < 0.05, ##P < 0.01 compared with PHA; n = 5 rats. All values are denoted as means ± SE.
Moreover, chronic hypoxia led to more abundant adhesion of platelets in arterioles in rats compared with normoxic animals. Even in some small pulmonary vessels the platelet deposition was observed more obviously in hypoxic rats than in normoxic rats (Fig. 2, A and B). The 15-LO inhibitor restrained the platelet deposition in pulmonary vasculatures in the hypoxic rats (Fig. 2, A and B).

A general decrease in pulmonary vascular branching and complexity, characteristics of the obliteration in the vessels, was observed in pulmonary vasculatures of hypoxic rats compared with normoxic rats. NDGA administration in the hypoxic rats recovered the diffused vascular blush (Fig. 2C).

The vWF, a platelet adhesive protein to the vessel wall and an indicator of endothelium dysfunction, was examined. We found that hypoxia raised the vWF expression in the small pulmonary arteries, which was reversed by NDGA (Fig. 2D).

However, we observed no significant difference in the staining of CD41 between hypoxic and normoxic groups in the

![Fig. 2. Coagulation was regulated by 15-LO pathway in and MCP1-dependent manner. A: coagulation time with 10^6 platelets was detected from normoxia, hypoxia, and hypoxia with NDGA rats. B: D-dimer concentrations were measured by ELISA. C: protein expression of factor Xa was assessed in normoxia, hypoxia, and hypoxia with NDGA rats. D: Activated fibrinogen receptors (D) and platelet cell-surface P-selectin (E) were examined by FACScan flow cytometer (Becton Dickinson). The concentrations of β-TG (F) and PF4 (G) in the plasma were determined by ELISA (BioTek). Nor, normoxia; Hyp, hypoxia; N, NDGA; B, bindarit. *P < 0.05; n = 5 rats. All values are denoted as means ± SE.](downloaded_from_http://ajplung.physiology.org.org)
systemic arteries, including the aorta, the common carotid artery, and the mesenteric artery (Fig. 3, A–C). Meanwhile, there was no significant difference in the platelet deposition of the small artery between hypoxic and normoxic groups in the organs or tissues of systemic circulation including the heart (Fig. 3D), liver (Fig. 3E), kidney (Fig. 3F), spleen (Fig. 3G), and quadriceps femoris muscle (Fig. 3H). These results demonstrated that the platelet deposition induced by hypoxia existed in the pulmonary circulation specifically.

Involvement of the 15-LO pathway in the hypercoagulable state of blood. To demonstrate the role of 15-LO pathway in the coagulation in vitro, we administrated the exogenous 15-HETE to the platelet of the whole blood. Our results showed that with identical platelet counts, the coagulation time was significantly reduced after exogenous 15-HETE (5 μM) treatment for 0.5 h (Fig. 4A). Fibrin D-dimer is a degradation product of cross-linked fibrin, negative detection of which is regarded as a marker of exclusion test in patients with suspected pulmonary embolism (5). We found that exogenous 15-HETE could elevate the D-dimer concentrations induced by hypoxia, which was suppressed by the 15-LO inhibitor (Fig. 4B). Factor Xa, as part of the prothrombinase complex, catalyzes thrombin generation, leading to fibrin deposition. Factor Xa is critical for effective blood coagulation, as evidenced by the severe bleeding phenotype of FX-deficient individuals (30) and the embryonic or perinatal lethality exhibited by FX−/− mice (43). Factor Xa inhibitor, such as rivaroxaban and enoxaparin, has been shown to effectively reduce the incidence of thrombosis in patients undergoing orthopedic surgery without significantly increasing bleeding (11). Previous reports have shown that factor Xa plays a role in experimental PAH (8). We found that hypoxia enhanced the protein expression of factor Xa, which was also restrained by the siRNA of 15-LO1 or 15-LO2 (Fig. 4C).

The platelet activation was associated with 15-LO pathway. Platelet activation marked by activated fibrinogen receptor was reported to be correlated to the severity of PAH secondary to sickle disease (46). We found that the percentage of platelets positive for PAC-1, representing activated fibrinogen (activated GPIIb/IIIa) receptors, increased in the plasma with 15-HETE administration (Fig. 5A). The 15-LO inhibitor NDGA reversed the increased activated platelets by ADP. Meanwhile, the markers of platelet α-granule release, such as the surface expression of P-selectin, and the concentrations of β-TG and PF4 in the plasma were also increased by 15-HETE. Their increased expression and concentrations induced by ADP were restrained by NDGA (Fig. 5, B–D).

MCP-1, IL-6, and 15-LO in PBMCs. Several proinflammatory cytokines were examined. As shown in Table 1, 15-HETE content was correlated positively with the levels of MCP-1 (r = 0.68, P = 0.001) and IL-6 (r = 0.46, P = 0.03), which seems specific since none of IL-1β, IL-8, TGF-β, or TNF-α was correlated to 15-HETE levels. To investigate the relationship between the two cytokines and the 15-LO/15-HETE pathway, we knocked down the gene expression of MCP-1 and IL-6, using siRNAs (the efficiency of transfection was shown in Fig. 6, A and B). The protein expression of 15-LO1 and 15-LO2 induced by hypoxia was not affected by MCP1 or IL-6 siRNA in PBMCs (Fig. 6, C–D).

The stimulation of PBMC by phytohemagglutinin (PHA) increased the proinflammatory cytokine levels (32, 34). After separate silencing of the 15-LO1 and 15-LO2 genes (the efficiency of transfection is shown in Fig. 7, A and B), the PHA-induced expression of MCP-1 was decreased, while the IL-6 protein expression was not affected by the 15-LO1/2 siRNAs. Meanwhile, the 15-LO inhibitors NDGA or CDC also reverse the MCP-1 expression but not the IL-6 expression in hypoxic PAH rats (Fig. 7, C and D).

MCP-1 and 15-LO regulated coagulation and platelet activation in vivo. To demonstrate the role of 15-LO and MCP-1 in the coagulation and platelet activation induced by hypoxia, the D-dimer concentrations, the expression of factor Xa, the activated GPIIb/IIIa, the surface expression of P-selectin, as well as...
as the β-TG and PF4 concentrations were examined. The D-dimer concentration elevated by hypoxia was suppressed by the 15-LO inhibitor or MCP-1 synthesis inhibitor bindarit in rats (Fig. 8, A and B). We found that hypoxia promoted the protein expression of factor Xa, which was also restrained by the 15-LO inhibitor or MCP-1 synthesis inhibitor (Fig. 8C). The activated GPIIb/IIIa and the surface expression of P-selectin upregulated by hypoxia were reversed by 15-LO inhibitor or MCP-1 synthesis inhibitor in rats (Fig. 8, D and E). The hypoxia induced β-TG and PF4 concentrations in rats were suppressed by 15-LO inhibitor or MCP-1 synthesis inhibitor (Fig. 8, F and G).

The coagulation regulated by 15-LO was associated with MCP-1. To determine the role of MCP-1 in the coagulation regulated by 15-LO, the D-dimer concentrations and the expression of factor Xa were examined. The content of D-dimer induced by hypoxia was significantly reduced by silencing the gene of MCP1. The exogenous 15-HETE augmented the D-dimer concentrations, which was reversed by siMCP-1 (Fig. 9A). Meanwhile, we observed similar phenotypes in which the expression of factor Xa induced by hypoxia was decreased by knocking down the gene of MCP1. The increased factor Xa expression induced by exogenous 15-HETE was inhibited by siMCP-1 (Fig. 9B).

**MCP-1 in 15-LO-mediated platelet activation.** To assess whether MCP-1 was associated with the platelet activation regulated by 15-LO pathway, the activated GPIIb/IIIa, the surface expression of P-selectin, as well as the β-TG and PF4 concentrations were detected. Administering the anti-MCP-1 antibody reduced the GPIIb/IIIa activated by ADP, which was reversed by exogenous 15-HETE (Fig. 10A). Similarly, a significant decrease in the P-selectin and PF4 was observed after reducing the protein expression of MCP-1, whereas the effect was reversed by the exogenous 15-HETE (Fig. 10, B–D).

**DISCUSSION**

In this study we find evidence for the role of 15-LO/15-HETE signaling in the thrombus formation in PAH pulmonary vasculatures. The 15-LO pathway mediates the endothelium dysfunction, platelet deposition, and thrombosis in...
vivo. Meanwhile, the 15-LO pathway regulates the coagulation and platelet activation in which proinflammation cytokines play a role.

The lipoxigenases (LOs) consist of 5-, 8-, 12-, and 15-LOs in mammals. In the latter, there are two members, 15-LO1 and 15-LO2, both of which are expressed in different cell types including macrophages, smooth muscle cells, and endothelial cells. The 15-LOs convert arachidonic acid to 15-HETE (7, 31, 40, 51). Macrophage 15-LO are involved in the development of experimental hypertension (22) and atherosclerosis (18). The endothelial 15-LO overexpression enhanced atherogenesis (16). In this study we find that 15-HETE reduces the blood coagulation time and induces platelet activation, similar to the previous report that 15(S)-HETE strongly prevents thrombin induced-platelet aggregation inhibition (4).

The endothelial cells produce and release vWF, which functions as an adhesive protein in the interaction of platelets with the vessel wall and is a useful marker of endothelial injury or dysfunction. Increased expression has been described in vWF levels and activity in patients with PAH (13, 36). In this study, we have found that hypoxia raises the vWF expression in the small pulmonary arteries which can be restrained by 15-LO inhibitor NDGA, suggesting a clue that 15-LO pathway is attributable to the endothelial dysfunction in pulmonary vessels. Meanwhile, 15-HETE shortens the coagulation time significantly (Fig. 4A), similarly to the 20-HETE effect reported to be correlated with shorter tail bleeding time and increasing rat platelet aggregation (24).

The thrombotic occlusion of small vessels by platelet aggregates contributes to the increase in pulmonary pressures (33). We have shown extensive platelet deposition indicated by CD41 staining in the pulmonary small arteries and arterioles of SuHx and hypoxic rats. This is not seen in the systemic vasculatures of the same hypoxic rats. Although hypoxia seems to lead to the blood hypercoagulable state, the specific endothelial dysfunction in the lung vessels induced by hypoxia determines that the hypercoagulable blood is liable to form thrombi in pulmonary vasculature.

Inflammation is accompanied with thrombosis in PAH and inflammatory cell infiltrates have been found in plexiform lesions of PAH (10, 19, 44, 45). Elevated proinflammatory cytokines activity and their increased plasma levels have been reported in rat monocrotaline and hypoxia models of PAH compared with control subjects (3, 35). We detect some cytokines and growth factors in the plasma from PAH patients. Our analysis of the correlation of 15-HETE with the cytokines indicates that MCP-1 and IL-6 are regulated by 15-LO1/2 in PBMCs. The 15-LO/15-HETE signaling is also found to up-regulate the expression of several cytokines such as IL-6, IL-8, MCP-1, and TNF-α in atherosclerosis, heart failure, and severe malaria in several previous studies, in which vascular inflammation seems to occur as well (6, 14, 20, 49).

Endothelial cell dysfunction with deregulated expression of proinflammatory mediators may account for different and characteristic lesions associated with PAH, such as in situ thrombosis, pulmonary arteritis, and typical plexiform lesions with their glomoidlike exuberant endothelial cell proliferation (25, 26). Transmembrane protein of activated platelets induced the inflammation by promoting the upregulation and release of IL-8 and MCP-1 chemokines, leading to lung perivascular invasion by macrophages and lymphocytes (45). The observed platelet activation may explain the enhanced generation of 15-HETE, because platelets are a partial source of 15-LO (50).

In conclusion, our results suggest a new signaling pathway of the 15-LO/15-HETE in endothelium dysfunction, platelet activation, and blood coagulation in pulmonary arteries. Targeting at the signaling system may be a novel strategy for treatment of PAH. Thus these findings may have important implications for the understanding and treatment of PAH.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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