Cellular mechanisms and intracellular signaling pathways for the modulation of eNOS in pulmonary arteries by 15-HETE

Lei Yu1*, Yun Liu1*, Zhaoping Qiu1, Shulin Liu2, Xu Gao3, and Daling Zhu1,4

1Department of Biopharmaceutical Sciences, College of Pharmacology, Harbin Medical University, Nangang District, Harbin, Heilongjiang, P. R. China, 2Genomic Research Center, Harbin Medical University, Harbin, P. R. China, 3Department of Biochemistry and Molecular Biology, Harbin Medical University, Harbin, P. R. China, and 4Department of Biopharmaceutical Sciences, College of Pharmacy, Harbin Medical University (Daqing), Daqing, P. R. China

Abstract
The 15-hydroxyeicosatetraenoic acid (15-HETE), a lipid metabolite and vasoconstrictor, plays an important role in hypoxic contraction of pulmonary arteries (PAs) through working on smooth muscle cells (SMCs). Previous studies have shown that vascular endothelium is also involved in PAs tone regulation. However, little is known as to how the pulmonary artery endothelial cells (PAECs) are related to the 15-HETE-induced vasoconstriction and that which intracellular signaling systems are critical. To test this hypothesis, we examined PAs constriction in isolated rat PAs rings, the expression and activity of endothelial nitric oxide synthase (eNOS) with western blot, and nitric oxide (NO) production using the DAF-FM DA fluorescent indicator. The results showed that the 15-HETE-induced PAs constriction was diminished in endothelium-intact rings. In the presence of the eNOS inhibitor L-NAME, vasoconstrictor responses to KCl were greater than the control. The activation of eNOS was activated by Ca2+ released from intracellular stores and the PI3K/Akt pathway. Phosphorylations of the eNOS at Ser-1177 and Akt at Ser-473 were necessary for their activity. A prolonged 15-HETE treatment (30 min) led to a decrease in NO production by phosphorylation of eNOS at Thr-495, leading to augmentation of PAs constriction. Therefore, 15-HETE initially inhibited the PAs constriction through the endothelial NO system, and both Ca2+ and the PI3K/Akt signaling systems are required for the effects of 15-HETE on PAs tone regulation.

Keywords: 15-hydroxyeicosatetraenoic acid, pulmonary arteries, nitric oxide, endothelial nitric oxide synthase

Introduction
The constriction of pulmonary arteries (PAs) in the hypoxic zone is essential for the ventilation–perfusion balances in lungs. Both pulmonary artery endothelial cells (PAECs) and smooth muscle cells (SMCs) are involved (1). We have previously shown that subacute hypoxia upregulates activity of 15-lipoxygenase (15-LO) that converts arachidonic acid to 15-hydroxyeicosatetraenoic acid (15-HETE). Exogenous 15-HETE produces strong constriction of rabbit PAs rings that have been exposed to chronic hypoxia, while the effect is much lower in normoxic condition, suggesting that 15-HETE produced in hypoxia may serve as a regulator of PAs tones (2). Previous studies have shown that endothelium, which was made of small population of endothelial cells (ECs) in a tissue, has a major homeostasis function in tissue and regulates vascular tone via release of endothelium-derived relaxing factors and endothelium-derived hyperpolarizing factor (3–7). Although the involvement of vascular SMCs in the process has been reported, how endothelia act in the 15-HETE-induced PAs constriction is still unclear.

Nitric oxide (NO) synthesized from the amino acid L-arginine by the NO synthases is the major vasodilator released from endothelium of small vessels and plays an important role in the regulation of vascular tones (1). Several previous reports have shown that hypoxia attenuates the synthesis and release of NO from the PAs endothelium by certain unknown mechanisms (8,9). Experimental evidence indicates that the NO biosynthesis increases and the angiotensin

*These authors contributed equally to this work.
Address for Correspondence: Dr. Daling Zhu, College of Pharmacy, Harbin Medical University (Daqing), Xinyang Road, Daqing, Heilongjiang 163319, P. R. China. Tel: 011-86-459-8153555. Fax: 011-86-459-8153556. E-mail: dalingz@yahoo.com
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II-dependent vasoconstriction is impaired in 12/15-lipoxygenase (12/15-LO) knockout mice, suggesting a link of 12/15-LO with NO (10). The 15-LO is expressed in a variety of mammalian tissues, including PAECs and SMCs (4,11,12). The 15-LO activation involves Ca^{2+}-dependent membrane translocation (13,14). It is known that the endothelial nitric oxide synthase (eNOS) is activated by the phosphoinositide 3 kinase (PI3K) and protein kinase B (Akt) signaling system (15,16), for which eNOS phosphorylation at residue Ser-1177 (in human, Ser-1179 in bovine) is necessary (9,13,14,17). Of all Akt isoforms, the Akt-1 plays a prominent role in ECs, as the eNOS phosphorylation, NO release, and angiogenesis are drastically reduced in Akt-1-knockout mice (18). In addition to the PI3K/Akt pathway, eNOS is regulated by intracellular Ca^{2+}/calmodulin-dependent protein kinase (CaMK). Hypoxia causes a fast increase in intracellular Ca^{2+} in PAs endothelia, owing to the Ca^{2+} release from intracellular stores (14,19,20). The rise in intracellular Ca^{2+} subsequently leads to Ser-1177 phosphorylation and augmentation of eNOS activity (13,14,21), which is likely to be due to the binding of eNOS to CaMK and Akt as shown in immunoprecipitation (21–23). Dephosphorylation of the eNOS at residue Thr-495 also enhances their association and increases eNOS activity (21).

As an important hypoxic product converted through 15-LO, 15-HETE plays an important role in hypoxia-mediating PAs tone regulation. To determine what role the endothelia and the cellular mechanisms and intracellular signaling systems for the 15-HETE-induced PAs constriction plays, we performed these studies in isolated PAs rings and dissociated PAECs by exogenous 15-HETE. The results showed that the 15-HETE-induced PAs constriction was dampened in the presence of endothelia, owing to augmentation of eNOS activity by phosphorylation at Ser-1177 in which Ca^{2+} and PI3K/Akt play a role. The late effect of 15-HETE on endothelia was from phosphorylation at Thr-495 and decreased eNOS protein level.

Materials and methods

Reagent
The 15-HETE and nitro-l-argininemethyl ester (L-NAME) were purchased from Cayman Chemical (Ann Arbor, USA). The LY294002, 3-Amino, 4-aminomethyl-2′, 7′-difluorescein, diacetate (DAF-FM), and RIPA lysis buffer (strong) were obtained from Beyotime Institute of Bio-technology (Haimen, China). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham International (Amersham, UK). The eNOS and AKT1/2 polyclonal antibodies were bought from Santa Cruz Biotechnology Inc. Rabbit polyclonal antibodies against phosphorylated eNOS (Ser-1177), phosphorylated eNOS (Thr-495), and phosphorylated Akt (Ser-473) were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). The phosphatase inhibitors and protease inhibitors tablet were acquired from Roche Applied Science. All other reagents were purchased from Sigma-Aldrich Co (St. Louis, USA).

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 PAs isolated from calf lungs. The arteries were slit open along their lengths and gently scratched along the intimal surface with a surgical blade and washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer to remove blood. A collagenase solution (0.5 mg/mL in DMEM) was prepared and filtered through 0.22 µm filter before use. The cells were immediately transferred to a 15 mL centrifuge tube, and incubated at 25°C for 8–9 min. Then the cells were vortexed and centrifuged at 1500 rpm for 10 min to obtain cell pellets. After the supernatant was discarded, the cell pellets were rinsed three times. The cells were resuspended in 5–7 mL culture medium (DMEM containing 20% fetal bovine serum [FBS]), plated in the 50 mL cell culture flask, and cultured in a 5% CO_{2} incubator at 37°C. The purity and identity of PAECs were confirmed by positive immunofluorescence staining using antibodies to CD31 (Santa Cruz, USA). The cells were used for experiments at passages 4–6.

Measurement of NO production in PAECs
The NO productions in cultured ECs were studied using the DAF-FM fluorescent indicator for intracellular NO (24). The ECs were grown on a microcoverglass (Matsunami Glass) of the 100 mm culture dish to ~80% confluence. The cells were first loaded with DAF-FM (final concentration, 5 µM) for 20 min at 37°C, rinsed three times in phosphate-buffered saline (PBS), and then used for experiments. The cells were maintained in a dark environment at 37°C. The microscopic fluorescent imaging systems resembled the ones described previously. The NO production was visualized by emission of green light (515 nm) upon excitation at 489 nm over a period of up to 9 min (25).

Tension studies of PA rings
Adult female/male Wistar rats with a mean weight of 200 g were obtained from the Experimental Animal Center of Harbin Medical University (Grade II), China. The animals were conditioned at a controlled ambient temperature of 22 ± 2°C with 50 ± 10% relative humidity and at a 12 h light-dark cycle (lights on at 8:00 AM). Standard rat chow and water ad libitum were provided to all rats. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Harbin Medical University. We anesthetized each rat with pentobarbital injection (120 mg/kg, i.p.), opened the thorax, and removed the heart and lungs to the flat plate. The lungs were put into chilled HEPES buffer (NaCl 138, KCl 4, CaCl\textsubscript{2} 1.6, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, EDTA-Na\textsubscript{2} 0.026,
Glucose 6, HEPES 10, in mmol/L, PH 7.4, with 95% O2 and 5% CO2) MgSO4 0.57, KH2PO4 1.2, NaHCO3 20, EDTA-Na2 0.02, Glucose 10, in mmol/L, PH 7.4, with 95% O2 and 5% CO2) at 37°C. Tension of 0.3 g was gradually and incrementally applied over 30 min and then equilibrated for an additional 30–40 min at 37°C. Tension data were measured with force-electricity transducers, acquired with a signal amplifier (600 series eight-channel amplifier, Gould Electronics), and collected with CODAS software (Data Q Instruments, Inc.).

Protein preparation/western blotting
The PAECs were cultured in a 6-cell dish. When cells were grown to 90% confluence, they were incubated with or without 15-HETE (1 µM) (26) and Ca2+-free, Ca2+-free with 15-HETE, LY294002 (10 µM), LY294002 with 15-HETE (1 µM) for indicated times. Then cells were chilled on ice and washed three times with cold PBS. They were scraped in presence of 100 µL RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin) supplemented with phosphatase inhibitors (PhosSTOP Phosphotase Inhibitor Cocktail Tables, Roche) and protease inhibitor (Complete Protease Inhibitor Cocktail Tables, Roche). The mixture was collected in an eppendorf tube. After equilibration on ice for 10 min, lysates were centrifuged for 10 min at 12,000 g, and the supernatants were collected to determine protein concentration via the BioRad protein assay kit. Proteins in the cell supernatant were heated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 5 min and separated by 8% SDS-PAGE as described previously (27). After the proteins were transferred to nitrocellulose membrane, the membrane was blocked with 5% nonfat dried milk for 1 h at room temperature. Membranes were incubated overnight at 4°C in tris-buffered saline and Tween 20 (TBS-T) containing BSA and primary antibodies, such as anti-eNOS (1:1000 dilution), anti-phosphorylated eNOS (Ser-1177, 1:1000 dilution), and anti-phosphorylated eNOS (Thr-495, 1:1000 dilution). After the membrane was washed six times in TBS-T about 40 min, the corresponding secondary antibodies were added for 1 h. Following three washes, the samples were visualized by enhanced chemiluminescence.

To probe western blots with alternative primary antibodies, the nitrocellulose membranes were washed with TBS-T for 5 min, followed by a 5 min rinse with the stripping buffer. Then, the membranes were blocked with 5% nonfat dried milk for 1 h at room temperature and subsequently treated with the primary antibodies. Further steps were similar to above. Densitometry was accomplished by using Quantity One quantitative gel software.

Statistical analysis
The experimental data are expressed as means ± SEM. Statistical analysis was made with one-way analysis of variance (ANOVA) followed by appropriate Dunnett’s test. Differences were considered to be significant if p ≤ 0.05.

Results
Effects of 15-HETE on endothelium-intact and endothelium-denuded PA rings
Isometric PAs contractions were studied in endothelium-intact (EI) and endothelium-denuded (ED) rings. Administration of exogenous 15-HETE led to slow constrictions of both EI and ED rings (Figure 1A). Such an effect was greater in ED rings than in EI rings, which manifested itself clearly in 5 min of the 15-HETE exposure, suggesting the involvement of endothelium-derived vasodilators. In comparison, the PAs contractile force was reduced by ~52% in the presence of endothelia (Figure 1B).

Involvement of NO in PA endothelia
Since NO is the major vasorelaxant in endothelia, we examined the contractile force of the PAs rings cultured in hypoxic condition for 24 h to release endogenous 15-HETE (28). The effect of NO was studied with NG-nitro-l-arginine methyl ester (l-NAME), a selective NOS inhibitor. In the EI rings, the PAs constriction induced by KCl was significantly enhanced with a 30 min exposure to l-NAME (10−5 mM, Figure 1C and 1D).

To test whether the NO production indeed changes with 15-HETE treatment, we measured the intracellular NO using the DAF-FM fluorescent indicator in cultured bovine PAECs. The ECs exposed to 15-HETE, and what intra-endothelial signaling systems play a role? To address these questions, we studied eNOS expression and activity in cultured PA ECs.

It is known that the protein phosphorylation at residue Ser-1177 increases eNOS activation, while phosphorylation at Thr-495 results in eNOS inhibition (21). Therefore, we examined phosphorylation levels at the two sites using phosphorylation-selective antibodies. The PAECs
were treated with the 15-HETE for different time periods (2, 5, 30, and 120 min). Evident increase in Ser-1177 phosphorylation was seen at 2–5 min with the 15-HETE stimulation. The Ser-1177 phosphorylation returned to the baseline level in 30 min (Figure 3A and 3B). The Thr-495 phosphorylation level showed detectable increase in 30 min and returned to normal in 120 min (Figure 3A and 3C). Taken together, these results indicate that the early effect of 15-HETE on NO production is likely to be mediated by augmentation of eNOS phosphorylation at Ser-1177, while phosphorylation at Thr-495 was found to inhibit NO production in 30 min. Interestingly, we found that eNOS protein level decreased within 30 min, which may take part in the NO production inhibition by 15-HETE (Figure 3A and 3D).

**Intracellular signaling systems for the eNOS and Akt phosphorylations**

As the eNOS activation in ECs is Ca²⁺-dependent (29), we examined whether the activity of eNOS required Ca²⁺ entry from the extracellular medium. We found that a removal of extracellular Ca²⁺ lowered the basal Ser-1177 phosphorylation significantly, but did not block the 15-HETE-induced Ser-1177 phosphorylation (Figure 4A and 4B), suggesting that the early effect of 15-HETE requires a rise in intra-endothelial Ca²⁺, in which the Ca²⁺ release from intracellular stores is crucial.

The PI3K/Akt pathway was also tested for the early effect of 15-HETE. Our results showed that the enhanced Ser-1177 phosphorylation was eliminated completely in the presence of the PI3K inhibitor LY294002 (10⁻⁵ mM, Figure 3A and 3C).

The eNOS is known to bind with Akt after the Akt is phosphorylated at Ser-473, leading to Akt activation (30). Thus, we analyzed the Akt phosphorylation at Ser-473 in PAECs with and without 15-HETE stimulation for 5 min. Similar to the Ser-1177 phosphorylation of the eNOS, the 15-HETE stimulation enhanced the Akt phosphorylation at Ser-473. The effect was retained after the removal of extracellular
Ca²⁺ (Figure 4A and 4E) but blunted in the presence of LY294002 (Figure 4A and 4F). These results, therefore, suggest that both the Ca²⁺ release from intracellular stores and the activation of the PI3K/Akt signaling pathway are necessary for the early NO release with the 15-HETE treatment.

**Figure 2.** Effect of 15-HETE on NO production in PAECs in 5 min and 30 min. Basal NO production is visualized with DAF-FM. PAECs were treated with absence or presence of 15-HETE. In 5 min, 15-HETE stimulated the release of NO product (Figure 2A and 2B) and diminished the NO release significantly after 30 min (Figure 2C and 2D).

**Figure 3.** Time course of the 15-HETE-induced phosphorylation and protein of eNOS. Representative western blots and summary data showing the effects of 15-HETE and eNOS Ser-1177 (Figure 3A and 3B), Thr-495 (Figure 3A and 3C), and total eNOS (Figure 3A and 3D). The 15-HETE induced Ser-1177 phosphorylation starting from 2 min, and lasting 5 min at least, and increase phosphorylation at Thr-495 within 30 min. The eNOS protein was inhibited by 15-HETE within 30 min. Results are presented as the mean ± SEM; of four separate experiments, *p < 0.05, **p < 0.01 compared with the respective control group.

eNOS modulation in a prolonged period of 15-HETE stimulation
The 15-HETE did not change the eNOS phosphorylation at Ser-1177 after a 30 min treatment; such a treatment was capable of maintaining the Ser-1177 phosphorylation after...
removal of extracellular Ca\textsuperscript{2+} (Figure 5A and 5B). The Ser-1177 phosphorylation was almost completely eliminated in the presence of the PI3K blocker LY294002 (Figure 5A and 5B). Similarly, a 30 min treatment of the PAECs with 15-HETE also reserved the Ser-473 phosphorylation of Akt in Ca\textsuperscript{2+}-free condition, and the effect was critically dependent on the PI3K/Akt pathway (Figure 5A and 5C).

**Discussion**

The 15-HETE has been well studied as a vasoconstrictor released with chronic hypoxia (31). Its primary target is the vascular smooth muscle. In addition, the present study indicates that PAs endothelia plays an important role, as the vasoconstrictive effect of 15-HETE is significantly lower in EI rings than in ED rings.

The vascular endothelium releases various vasoactive substances, including NO, endothelin, EETs, etc. There is evidence that NO release is impaired in pulmonary hypertension, which has long been believed to underlie the pathogenesis of the disease (17,32). Furthermore, NO release is apparently decreased in chronic hypoxia. Our data from the present study have shown that 15-HETE has a biphasic effect on the NO production in cultured PAECs. The NO production is enhanced with a short (5 min) period of exposure but is suppressed in 30 min. The short period of effect of 15-HETE seems attributable to the attenuation of PAs constriction in EI rings, as its time course is consistent with that of PAs constriction. Also the L-NAME effect that inhibits eNOS, exaggerating the KCl-induced PAs constriction is consistent.
Our data also suggest that the transient effect of 15-HETE on NO production is mediated by enhanced eNOS activation. The endothelial effect seems to depend on post-translational modulation of the eNOS, consistent with the notion that eNOS activity not only depends on the enzyme abundance but also is regulated via phosphorylation (33,34). Several phosphorylation sites in the eNOS have been shown. Phosphorylation at residues Ser-617, Ser-635, Ser-1177, and Ser-1179 results in eNOS activation, whereas phosphorylation of Ser-116, Thr-495, and Thr-497 causes inhibition of eNOS activity (21,35–37). Our results indicate that 15-HETE induces eNOS phosphorylation at Ser-1177 with the time course consistent with endothelial effect on PAs constriction and also inhibits NO production in PAECs through phosphorylation at Thr-495.

Interestingly, protein level of eNOS does not change with a short period of 15-HETE exposure, while it decreases with a prolonged 15-HETE treatment of 30 min. The decrease in eNOS protein expression indicate that the effect of 15-HETE may also decrease the steady state levels of eNOS mRNA. It is likely that the changes are due to reduction in the transcription rate. In such a short period of time, 15-HETE decreased the expression of eNOS but the mechanism is not clear.

The Akt is a serine/threonine kinase activated by several phosphatidylinositol-dependent protein kinases. Phosphorylation of Ser-473 on Akt is coincident with Akt activation in vivo and in vitro and has been used as a marker for Akt activity. Kinase Akt (or protein kinase B) is an important determinant of eNOS phosphorylation at Ser-1177, implying intimate involvement in basal activation of eNOS and agonist-mediated stimulation (14,27,38,39). Our results indicate that 15-HETE also acts on the PI3K/Akt pathway to enhance eNOS activity for several reasons (1): The enhanced Ser-1177 phosphorylation is almost completely eliminated in the presence of PI3K/ Akt inhibitor (2); the Akt phosphorylation at Ser-473 is stimulated by 15-HETE and blocked by its inhibitor; and (3) the Akt phosphorylation at Ser-473 is consistent with that of the Ser-1177 phosphorylation. The Ser-1177 phosphorylation is also Ca2+-dependent. Its phosphorylation level decreases in Ca2+-free condition. Although extracellular Ca2+ plays a role, Ca2+ entry is not the only source. Indeed, Ca2+ release from the intracellular store (20) seems more important for the 15-HETE effect on Ser-1177 phosphorylation, as 15-HETE upregulates the Ser-1177 phosphorylation in Ca2+-free condition as potently as with extracellular Ca2+. The Ca2+ signal is not only required for Ser-1177 phosphorylation but also is important for Akt phosphorylation at Ser-473. Therefore, both the activation of PI3K/Akt pathway and the Ca2+ release from the intracellular store are crucial for the transient effect of 15-HETE on the NO system of PA endothelia.

With a prolonged exposure to 15-HETE or chronic hypoxia, the eNOS protein level decreases as shown in our results as well as previous studies (8). Prolonged
15-HETE exposure dramatically decreases the eNOS protein level in 30 min, suggesting that this decrease is likely from protein degradation. Further studies will be needed to clarify this issue. In conclusion, the effects of 15-HETE on PAs vasoconstriction involve both vascular smooth muscles and endothelia. It acts on the NO system in endothelia transiently and lowers its PAs vasoconstriction effect, which may occur under normal physiological condition without leading to extensive PAs constriction. The attenuation of PAs constriction by endothelia disappears with a prolonged exposure to 15-HETE, augmenting markedly the PAs vasoconstriction effect of 15-HETE, which is likely to take place during chronic hypoxia. The short period regulation of the endothelial NO system appears to be mediated by eNOS phosphorylation at Ser-1177 in which the activation of the PI3K/Akt pathway and the Ca\(^{2+}\) release from the intracellular store seems critical. The long-term effect (>30 min) is likely to result from the phosphorylation at Thr-495 and the decrease of eNOS protein. The demonstration of endothelial contribution to the 15-HETE effects and the intracellular signaling systems may not only help in understanding the vascular physiology but also in understanding the design of effective therapies for the treatment of pulmonary hypertension.

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Declaration of interest

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