15-hydroxyeicosatetraenoic acid depressed endothelial nitric oxide synthase activity in pulmonary artery

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Abstract: 15-hydroxyeicosatetraenoic acid (15-HETE) plays an important role in hypoxia-induced pulmonary vasoconstriction. Release of nitric oxide (NO) is apparently decreased and activity of endothelial nitric oxide synthase (eNOS) is impaired in chronic hypoxia. However, little is known whether 15-HETE contributes to eNOS/NO pathway in the constriction induced by 15-HETE. We examined the response of rat pulmonary artery (PA) rings to 15-HETE, the production of NO, total eNOS expression and the phosphorylation of eNOS in bovine pulmonary artery endothelial cells (BPAECs) stimulated by 15-HETE. Rat PA rings were divided into three groups: endothelium intact group, endothelium denuded group, and nitro-L-arginine methyl ester (L-NAME, 0.1 mmol/L, an inhibitor of eNOS) group. Constrictions to 15-HETE were significantly enhanced in endothelium denuded group and L-NAME group (both P<0.05 vs endothelium intact group, n=9); BPAECs were incubated in different conditions to test nitrite production by Greiss method. Nitrite production was significantly reduced by 1 µmol/L 15-HETE (P<0.05), and increased by the lipoxygenase inhibitors, 10 µmol/L cinnamyl 3,4- dihydroxy-[alpha] -cyanocinnamate (CDC, P<0.05) and 0.1 mmol/L nordihydroguaiaretic acid (NDGA, P<0.01 ); Western blot analysis of extracts from BPAECs incubated with 15-HETE in different time was carried out to test total eNOS expression, and the expression was changed unobviously. Immunoprecipitation (IP) and Western blot analysis of cell extracts from BPAECs treated with 2 µmol/L 15-HETE in different length of time were accomplished, using phospho-eNOS-threonine 495 (Thr495, an inhibitory site) antibody for IP, and eNOS or 15-lipoxygenase (15-LO) antibodies for Western blot. 15-HETE depressed eNOS activity by increasing the levels of phospho-eNOS-Thr 495. The data suggest that eNOS/NO pathway is involved in PA constrictions induced by 15-HETE and that 15-HETE depresses eNOS activity by phosphorylation in Thr495 site. The protein interaction between phospho-eNOS (Thr495) and 15-LO is discovered for the first time.

Key words: 15-hydroxyeicosatetraenoic acid; 15-lipoxygenase; pulmonary artery vasoconstriction; endothelium cells; endothelial nitric oxide synthase

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Hypoxic pulmonary vasoconstriction (HPV) is essential to balance perfusion with ventilation and is unique to pulmonary circulation, but its mechanism is complicated and not very clear. The mechanism of HPV is related to not only pulmonary artery (PA) endothelial cells (PAECs) but also pulmonary artery smooth muscle cells (PASMCs)[1]. Zhu et al. recently reported that chronic hypoxia increased the production of 15-lipoxygenase (15-LO) in PAECs, leading to an elevated level of 15-hydroxyeicosatetraenoic acid (15-HETE), and induced stronger constriction of PA to 15-HETE compared with normoxia, indicating that 15-HETE may be a mediator of HPV[2]. Our research group has recently demonstrated that 15-HETE inhibited voltage-gated K+ (Kv) channels[3], and raised [Ca2+]i in PASMCs[4]. These data support that 15-HETE is the mediator of HPV. However, little is known whether 15-HETE could regulate the function of PAECs in HPV. In chronic hypoxia, it has been reported that eNOS activity in PAECs is down-regulated, and NO production decreased[5]. Moreover, 15-LO can be induced by interleukin 4 (IL-4) in HPV in pulmonary vein[2]. Peter et al. accompanied by a decrease in eNOS level in PAEC of HPV[2, 5]. Our research group has recently demonstrated that 15-HETE inhibited voltage-gated K+ (Kv) channels[3], and raised [Ca2+]i in PASMCs[4]. These data support that 15-HETE is the mediator of HPV. However, little is known whether 15-HETE could regulate the function of PAECs in HPV. In chronic hypoxia, it has been reported that eNOS activity in PAECs is down-regulated, and NO production decreased[5]. Moreover, 15-LO can be induced by interleukin 4 (IL-4) in HPV in pulmonary vein[2]. Peter et al. accompanied by a decrease in eNOS level in PAEC of HPV[2, 5].

1.2 Preparation of PA rings

PA rings were prepared according to the method published in previous studies[10, 11]. Briefly, the rat heart and lungs were removed en bloc after anesthetized with 10% chloral hydrate. The lungs were placed into iced HEPE’S buffer (NaCl 138, KCl 4, MgSO4 1.2, KH2PO4 1.2, EDTA-Na2 0.026, glucose 6, HEPES 10, in mmol/L, pH 7.4). Connective tissue and adipose tissue around PA were removed carefully, and the main stem arteries (1~1.5 mm in diameter) were cut into 3-mm long rings. Then the rings were mounted on to a tungsten wire, one end of which was connected to a fixed holder and the other to a force displacement transducer for PA rings isometric tension measurement, and the rings were immersed in pH-adjusted, oxygenated Kreb solution (NaCl 118, KCl 4, CaCl2 1.6, MgSO4 1.2, KH2PO4 1.2, EDTA-Na2 0.026, glucose 6, HEPES 10, in mmol/L, pH 7.4). Tension was relayed from pressure transducer to signal amplifier (Medlab 6.1 four-channel amplifier, MedEase). The rings were initially loaded with 0.3 g of tension that was gradually and incrementally applied over 30 min and then equilibrated for an additional 30 ~ 40 min in Krebs before the studies began.

1 MATERIALS AND METHODS

1.1 Materials and instruments

15-HETE, nitro-L-arginine methyl ester (L-NNAME), cinnamyl 3, 4- dihydroxy-[alpha]-cyanocinnamate (CDC), nordihydro- guaiaretic acid (NDGA) were all purchased from Cayman Chemical Co., Inc. Bovine serum albumin (BSA), Tween-20, phenylmethylsulfon fluoride (PMSF), leupeptin and aprotonin were from Sigma. Phospho-eNOS (Thr495) and eNOS antibodies (anti-phospho-eNOS or anti-eNOS, both from Cell Signaling Company), goat anti-rabbit IgG/HRP (TBD Science), NO assay kits (Beyond MedLab 6.1 four-channels amplifier was purchased from Nanjing MedEase Instruments Company.

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1. 3 PA rings experiment and grouping

The rings were suspended in organ baths containing 4 ml Krebs supplied with 95% O₂ and 5% CO₂ at 37°C. After equilibration for 30 min, 15-HETE from a stock solution (0.3 mmol/L) in ethanol was added to the baths at the final concentration 1 and 2 µmol/L at 10 min intervals as control group. After washed at least 3 times and equilibrated for 30 min, the rings of control group were preincubated with L-NAME for 30 min, and then added 15-HETE (1 and 2 µmol/L) in turn as L-NAME group. The changes in isometric contraction of the PA rings to 15-HETE from both L-NAME and control groups were recorded on MedLab 6.1 four-channel amplifier (MedEase).

In another experiment [12], PA rings were denuded of endothelium by passing through the lumens with a rough wire to and fro 20 times as endothelium denuded group, while rings was not so treated as endothelium intact group. Removal of the endothelium was verified routinely by the absence of ACh (1 µmol/L)-induced relaxation in PA rings precontracted with noradrenalin (NE, 10 µmol/L). The changes in isometric contraction to 15-HETE (1 and 2 µmol/L) were recorded.

1. 4 PAECs culture

PAECs were scraped from PA of neonatal bovine and cultured in DMEM with 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin in 30 mm dishes in a humidified atmosphere at 37°C. Cell viability (usually greater than 98%) was determined by Trypan blue exclusion. PAECs after 4–6 passages were used for NO production assay, IP and WB analysis.

1. 5 Nitrite analysis

Nitrite production in BPAECs was measured using a colorimetric assay on the basis of the method of Griess [13]. Cells were grown in 96-well plates in DMEM without phenol red, supplemented with 0.1% BSA. At confluence, different combinations of agents (1 µmol/L 15-HETE, 10 µmol/L CDC and 0.1 mmol/L NDGA, respectively) were added for 30 min, and 50 µl of the incubation medium were withdrawn for determination of nitrite concentration. Fifty microlitres of Griess reagent (1 part 1% sulfanilamide and 1 part 0.1% naphthylendiamine dihydrochloride in 5% phosphoric acid) was used. The absorbance was measured at 540 nm by a microtiter plate reader (Bio-Tek EL 340, Winooski, VT), and the nitrite concentration was determined using a calibration curve with NaNO₂ as a standard.

1. 6 PAECs lysis

The experiments were done as described previously[14]. Briefly, The PAECs were maintained in the different media (1 or 2 µmol/L 15-HETE) for the indicated period of time. Cells were at first solubilized in lysis buffer [20 mmol/L Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mmol/L NaCl, 2 mmol/L EDTA, 25 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, 2 mmol/L sodium pyrophosphate, 10 µg/ml leupeptin, and 1 mmol/L PMSF]. Then the cells were scraped off and dealt with ultrasonic processing for 20 s. After centrifuged at 14 000 r/min for 15 min at 4°C, the protein concentration of supernatant of the cell lysate was determined using the Coomassie blue protein assay (Bio-Rad Laboratories, Richmond).

1. 7 Western blot analysis

Cell lysates (30 µg protein/lane) were electrophoresed on an 8% SDS- polyacrylamide gel, and proteins were transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dried milk, and 0.1% Tween-20 for 1 h at room temperature. Membranes were incubated for 8 h at 4°C in TBST containing BSA and primary antibody, such as anti-eNOS (1:1 000 dilution) and anti-actin (1:1 000 dilution). After the membrane was washed three times in TBST, the corresponding secondary antibody, namely peroxidase-conjugated IgG, was applied for 90 min. After three washes in TBST, immunoreactive bands were detected using the enhanced chemiluminescence's detection system from Amersham (Arlington Heights, IL), according to the manufacturer's instructions.

1. 8 Immunoprecipitation analysis

Three hundred microgramme protein lysate of PAECs was diluted to 300 µl with lysis buffer, and then mixed with 40 µl Protein A-Sepharose (Amersham) at 4°C for 1 h. The samples were centrifuged at 1500 r/min for 5 min, and then the supernatant was aspirated carefully and incubated with anti-phospho-eNOS at 4°C overnight. Lysis buffer was added to wash the precipitation twice. Then proceed to WB analysis (the primary antibodies are eNOS or 15-LO antibodies, respectively).

1. 9 Statistical analysis

Values were reported as means ± SEM. Statistical comparisons of differences in the responses were conducted with the use of ANOVA, followed by Student- Newman-Keuls test or by WILCOXON followed by Kruskal-Wallis test. Differences were deemed significant at P<0.05.

2 RESULTS

2. 1 Role of endothelium in 15-HETE-induced constrictions in rat PAs

2. 1. 1 Vasoconstriction induced by 15-HETE was in-
increased by denuding PA endothelium
By detecting PA ring tension of endothelium in intact or denuded groups, we found that 15-HETE constricted rat PAs in either endothelium intact or denuded groups. Moreover the tensions induced by 15-HETE (2 µmol/L) in the endothelium-denuded group were obviously greater than that in the endothelium-intact group [(116.22 ± 4.97) %, n=7 vs (111.38 ± 6.20) %, n=9, P<0.05] (Fig. 1A). Tension of PAs from normoxia (data not shown) was not affected by 15-HETE in concentrations less than 1 µmol/L. Significant constriction in both endothelium intact or denuded groups was not observed (P>0.05).

2. 1. 2 Effect of L-NAME on PA vasoconstriction induced by 15-HETE
After incubation with L-NAME for 20 min, PA vasoconstrictions were induced by 15-HETE in different concentrations, especially when the concentration of 15-HETE was 2 µmol/L, and the constriction increased greatly in L-NAME group than in control group [(122.08 ± 10.69) %, n=9 vs (111.38 ± 6.20) %, n=9, P<0.05]). In addition, no significant difference was found between endothelium-denuded and L-NAME groups. These data suggest that eNOS/NO pathway might be involved in function of PAECs in PA constriction induced by 15-HETE (Fig. 1B).

2. 2 NO production assay
PAECs were treated with 15-HETE, CDC or NDGA, respectively, for 30 min, and then nitrite production in medium was examined. The results showed nitrite production decreased greatly in 15-HETE group (P<0.05 vs vehicle group), while nitrite production enhanced significantly in CDC and NDGA groups (P<0.05, P<0.01, respectively) compared with vehicle group (Fig. 2).

![Fig. 1. 15-HETE induced the constriction of rat pulmonary in different conditions. A: Effect of endothelium denuding on constriction of rat PA rings induced by 15-HETE. *P<0.05 vs endo-intact group, n=9. B: Effect of 0.1 mmol/L L-NAME on constriction of rat PA rings induced by 15-HETE. *P<0.05 vs control, n=9.](image)

![Fig. 2. Effect of 15-HETE and endogenous 15-HETE on eNOS activity in BPAECs. BPAECs were treated with 15-HETE, CDC, and NDGA, respectively, for 30 min. The results showed nitrite production decreased greatly in BPAECs treated with 15-HETE (*P<0.05 vs vehicle), while nitrite production enhanced significantly in CDC and NDGA groups (*P<0.05, **P<0.01, respectively vs vehicle).](image)

2. 3 Total eNOS assay
PAECs were treated with 1 µmol/L 15-HETE for 5, 10, 15, 20, 30 and 60 min, respectively, the total eNOS amount, namely eNOS expression, was detected by Western blot with anti-eNOS as primary antibody. No obvious difference was found in eNOS expression with different time length (Fig. 3), with actin as internal control.

2. 4 eNOS activity assay by eNOS antibody
PAECs were treated with 2 µmol/L 15-HETE for 30 and 60 min, respectively, the phospho-eNOS (Thr495) was immunoprecipitated by anti-phospho-eNOS (Thr495). Then anti-eNOS was used as Western blot primary antibody for detecting phosphorylation of eNOS at Thr495 site. As shown in Fig. 4, treatment with 15-HETE for 30, 60 min enhanced phosphorylation at Thr495 site.

2. 5 eNOS activity assay by 15-LO antibody
PAECs were treated with 2 µmol/L 15-HETE for 30, 60 and 120 min, respectively. The phospho-eNOS (Thr495) was immunoprecipitated by anti-phospho-eNOS (Thr495). 15-LO antibody was used as Western blot primary antibody for detecting phosphorylation of eNOS at Thr495 site. As shown in Fig. 5, phospho-eNOS (Thr495) was
found to be bounded with 15-LO for the first time, and treatment with 15-HETE for 30, 60 and 120 min enhanced phosphorylation of the Thr495 site.

3 DISCUSSION

HPV is an essential mechanism to balance perfusion with ventilation and is unique to the pulmonary circulation. The biochemical mechanisms which were hypothesized have multifactorial manifestations\textsuperscript{[15-17]}. But specific candidates that mediate HPV, such as NO, cytochrome P450 metabolites, leukotrienes, or direct effects of oxygen on ion channels in SMCs are unable to account for all the features of hypoxic vasoconstriction\textsuperscript{[16]}. HPV is intrinsic to the lung and is modulated upstream not only by the endothelium, but also by inhibited Kv channels including O$_2$-sensitive Kv channels, and by controlled Ca$^+$ influx in PASMCs\textsuperscript{[1]}. Recently, Zhu et al.\textsuperscript{[2]} reported that 15-HETE may serve as an essential mediator to link hypoxia and HPV. Based on this, our research group have demonstrated that 15-HETE not only inhibit Kv channels\textsuperscript{[3]}, but also raised [Ca$^+$]i in PASMCs\textsuperscript{[4]}. The results support the hypothesis proposed by Zhu et al\textsuperscript{[2]}. Dr. Zhu also reported the effect of 15-HETE on ion channels in PASMCs. In chronic hypoxia, it has been reported that eNOS activity in PAECs is down-regulated, and NO production decreased\textsuperscript{[5]}. If 15-HETE is a mediator of HPV, the effect of 15-HETE on regulation of eNOS should be the same as HPV. This paper focused on the influence of 15-HETE on eNOS/NO pathway, the signaling translation pathway in PAECs which maintains vascular tone and mainly is related to HPV.

The physiological significance of our research is that the results elucidated the mechanism of 15-HETE affecting eNOS/NO pathway in PAECs, for which exogenous 15-HETE depressed eNOS activity in PAECs exposed to normoxia. And endogenous 15-HETE could directly constrict pulmonary arteries. In addition, the protein interaction between phospho-eNOS (Thr495) and 15-LO was found for the first time. The results reported here support 15-HETE as an essential mediator to link hypoxia and HPV\textsuperscript{[2]}. Three pieces of information confirmed 15-HETE-induced inactivation of eNOS in PAECs. First, NO production assay demonstrated exogenous 15-HETE decreased the nitrite production in PAECs greatly, and endogenous 15-HETE enhanced the effect significantly. Second, WB results of PAECs' eNOS demonstrated 15-HETE did not affect the expression of eNOS distinctively. Third, WB results after IP of phosph-eNOS (Thr495) indicated 15-HETE up-regulated phosph-eNOS (Thr495).
In the study on PA ring tone, when the concentration of 15-HETE is 0.01 or 0.1 µmol/L, PA ring tension of all groups are not different significantly at basal tone (data not shown). But in HPV, 15-HETE in concentrations ≥ 0.01 µmol/L increased tension of PAs at basal tone [2]. In normoxia, 1 µmol/L 15-HETE started to increase tension of PAs at basal tone significantly. The data reported by Dr. Zhu support hypoxia enhances sensitivity of pulmonary arteries to 15-HETE[2]. 15-HETE of 2 µmol/L significantly increased the constriction of rings in both endothelium-denuded and L-NAME groups (vs endothelium-intact group), which means 15-HETE not only plays a role in rat PASMCs directly, but also affects eNOS/NO pathway in PAECs. The results indicate the constriction induced by 15-HETE is related to both PAECs and PASMCs.

NO is an essential endothelium-derived relaxation factor. The release of NO from PAECs appears to regulate vascular tone and counteract HPV[18]. Furthermore, NO release is apparently decreased in chronic hypoxia[46]. So we examined if 15-HETE affected the production of nitrite in PAECs. The results showed exogenous 15-HETE decreased NO formation, while CDC and NDGA, the inhibitors of 15-LO that inhibit the formation of endogenesis 15-HETE, increased the production of NO. In physiology condition in PAECs, NO was generated via L-arginine that is catalyzed by eNOS. L-arginine is not a limiting factor in production of NO by eNOS, so the expression and activation of eNOS is important in producing NO.

eNOS is an important enzyme in the pulmonary circulations. It catalyzes the production of NO, a key regulator of vascular tone, remodeling and angiogenesis[17, 19]. Our study showed the expression of total eNOS was not changed significantly when PAECs were treated with 15-HETE within 60 min, therefore the decrease of NO induced by 15-HETE could be resulted from down-regulation of eNOS activity. Activation of eNOS is, indeed, not only dependent on the enzyme abundance, site-specific phosphorylation, but also regulated through post-translational interactions with proteins (such as caveolin and the heat shock protein 90[27]). There are some clues hinting interactions between 15-LO and eNOS. First, 15-HETE, a product of 15-LO, was bound with phospho-eNOS (Thr495) for the first time, suggesting the possibility that eNOS activity could be influenced by 15-LO or its metabolite 15-HETE.

In summary, this study examined the effect of 15-HETE on eNOS/NO pathway for the first time. The results showed eNOS/NO pathway is involved in PA constrictrions induced by 15-HETE. 15-HETE depresses eNOS activity by phosphorylation at Thr495 site, and the interaction between phospho-eNOS (Thr495) and 15-LO may facilitate inactivation of eNOS.

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