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Reciprocal regulation of HIF-1α and 15-LO/15-HETE promotes anti-apoptosis process in pulmonary artery smooth muscle cells during hypoxia

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ABSTRACT

15-Hydroxyeicosatetraenoic acid, a predominant metabolic product of arachidonic acid (AA) catalyzed by 15-lipoxygenase (15-LO), plays an important role in hypoxic pulmonary arterial hypertension (PAH). Hypoxia-inducible factor-1α (HIF-1α) as a critical oxygen-sensitive transcriptional factor participates in many physiological and pathological processes including PAH. Therefore, it is possible that there may be some connections between HIF-1α and 15-LO/15-HETE in hypoxic pulmonary artery smooth muscle cells. Our results showed that HIF-1α inhibitor or siRNA reduced hypoxia-induced upregulation of 15-LO and endogenous 15-HETE, meanwhile HIF-1α expression and transcriptional activity were induced by 15-HETE under both normoxic and hypoxic conditions. It suggests there exists a potential positive feedback regulatory loop between HIF-1α and 15-LO/15-HETE. Furthermore, cell viability assay and several cell apoptosis assays, including TUNEL assay, Western blot, nuclear morphology determination, mitochondrial potential analysis, indicated that blocking HIF-1α induced apoptosis, decreased cell viability and suppressed the anti-apoptosis effects of 15-HETE. Taken together, our data indicate that upregulation of 15-LO/15-HETE in response to hypoxia may be partially mediated by HIF-1α which is also regulated by 15-HETE in a positive feedback manner, and HIF-1α can effectively inhibit pulmonary artery smooth muscle cells apoptosis which leads to vascular remodeling. The feedback loop between HIF-1α and 15-LO/15-HETE would obviously reinforce hypoxia-induced anti-apoptosis effect and may become a novel target of therapy in PAH.

1. Introduction

Pulmonary arterial hypertension (PAH) is a critical disease characterized by progressive increase resistance of the small pulmonary arteries as a result of vascular remodeling and sustained vasoconstriction, which leads to right heart failure and death [1,2]. Because of its high malignant potential the pathogenesis of PAH has always been a research focus for more than half a century. It has been reported that chronic hypoxic exposure induces the formation and development of PAH [3]. As an extended pathological alteration, vascular remodeling is mainly reflected in medial thickening. Therefore one of the most important events in remodeling process is that hypoxia prevents pulmonary artery smooth muscle cells (PASMCs) from apoptosis.

15-Hydroxyeicosatetrienoic acid (15-HETE) which participates in many physiological activities of cells is a predominant metabolite of arachidonic acid (AA) catalyzed by 15-lipoxygenase (15-LO) which has two isozyme types named 15-LO1 and 15-LO2 in hypoxic cells [4–6]. Our previous studies have shown that hypoxia activates 15-LO in pulmonary arteries to increase the production of 15-HETE, which leads to depolarization of PASMCs and pulmonary arterial vasoconstriction [7–11]. We have also found that both endogenous and exogenous 15-HETE inhibits PASMC apoptosis via several signaling pathways such as ERK1/2, PI3K/Akt, ROCK, etc. [12–14]. It demonstrates that 15-LO/15-HETE can promote the development of pulmonary vascular remodeling at the same time. Though 15-LO/15-HETE has been realized in recent years as an important factor in PAH, the mechanisms underlying how hypoxia up-regulates their levels is still unclear.

Hypoxia-inducible factor-1α (HIF-1α) has been recognized as a major regulator for cells and tissues to adapt hypoxia. After exposure of normal and cancer cells to hypoxia, amounts of HIF-1α protein stabilizes and accumulates rapidly in the nucleus where dimerized with HIF-1β [15,16]. And the production of HIF-1 leads to the transcriptional regulation of multiple genes that participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/apoptosis [17]. At present, it has been reported that HIF-1α is involved in the pathophysiology of hypoxic pulmonary hypertension. Partial HIF-1α deficiency significantly delayed the

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development of pulmonary vasoconstriction, vascular remodeling and hypertension compared with wild-type [18,19]. However, it is only revealed that HIF-1α can promote angiogenesis and PASMC hypertrophy to induce remodeling, its role in PASMC survival/apoptosis is not clear till now. We suspect that 15-LO/15-HETE may be one of the downstream factors of HIF-1α and participate in the processes of HIF-1α-induced pulmonary artery medial thickening via inhibiting cell apoptosis. To address these issues, we carried out this study. Our data suggest that HIF-1α can mediate the upregulation of 15-LO/15-HETE levels in hypoxia PASMCs, meanwhile, 15-HETE also induces HIF-1α expression and transcriptional activity via a positive feedback mechanism and this HIF-1α/15-HETE pathway can effectively inhibit PASMC apoptosis.

2. Materials and methods

2.1. Materials

15-HETE dissolved in ethanol was gained from Cayman Chemical Company (Ann Arbor, MI) and was stored at −20 °C under nitrogen. YC-1 was purchased from Alexis Biochemicals (San Diego, USA). 15(S)-HETE EIA Kit, 15-LO1 and 15-LO2 polyclonal antibodies were purchased from Cayman Chemical Company (Ann Arbor, MI). Antibodies against HIF-1α, procaspase-3, Bcl-2 and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). JC-1 probe and the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) cell apoptosis detection kit were gained from Beyotime Institute of Biotechnology (Haimen, China). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham International (Amersham, UK). All other reagents were from Sigma–Aldrich Co. (Missouri, USA).

2.2. Cell preparation

Adult male Wistar rats weighing 150–200 g were from the Experimental Animal Center of Harbin Medical University, which is fully accredited by the Institutional Animal Care and Use Committee (IACUC). PASMCs were prepared according to previously published protocol [20]. Cells were cultured in 20% fetal bovine serum (FBS)-DMEM of the condition of 37 °C, 5% CO_2. The purity of PASMCs in the primary cultures was verified by specific monoclonal antibodies raised against smooth muscle a-actin (Boehringer Mannheim, Germany). The cells were exposed to 3% O_2 in an incubator at 37 °C for hypoxic-induced. Passages 2–4 were used for further experimentations. Before each experiment, cells were synchronized via serum deprivation for 24 h. And the apoptosis in PASMC was still initiated by serum deprivation for another 24 h or 48 h.

2.3. MTT

PASMCs were cultured in 60-mm dishes (about 1×10^5), and then the cells were subject to growth arrest for 24 h before being placed in complete medium (DMEM with 20% FBS) for the next 48 h in normoxia or hypoxia environment. The samples were treated with exogenous 15-HETE, siRNA targeting HIF-1α or an unrelated siRNA respectively. At the end of the incubation period, 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 reaction was terminated by adding DMSO to the medium followed by incubation for 10 min at room temperature. Then the absorbance was read at 540 nm in a spectrophotometer.

2.4. Western blot analysis

Cultured PASMCs were initially treated with the chemicals for 24 h. Then, the cells were gently washed three times in cold PBS, and scraped into 300 μl lysis buffer (Tris 50 mM, pH 7.4, NaCl 150 mM, Triton X-100 1%, EDTA 1 mM, and PMSF 2 mM). The lysates were used for estimating the protein content with Bradford protein assay after the treatment of sonicate and centrifuged deposit. Samples containing 30 μg proteins were fractionated by 10% SDS-PAGE and then transferred to nitrocellulose membranes by electroblotting in a Mini Trans-Blot cell transfer apparatus (Bio-Rad) under conditions recommended by the manufacturer. After blocking for 1 h in room temperature with a Tris–buffered saline buffer (20 mM Tris, 150 mM NaCl, pH 7.6 Tween 20 0.1%) containing 5% nonfat dry milk, the membranes were incubated with appropriate antibodies to 15-LO1, HIF-1α at 1:1,000, 15-LO2, β-actin, procaspase-3, Bcl-2 at 1:500 dilution overnight at 4 °C. The membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution) or rabbit anti-mouse IgG (1:5000 dilution) antibodies for 1 h in room temperature. The bound antibodies were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham Biosciences, Piscataway, NJ) and exposed to X-ray film.

2.5. Mitochondrial depolarization assay

PASMCs were cultured in six-well plates and treated with reagents for 24 h under hypoxia. Then the treated cells were incubated with an equal volume of JC-1 staining solution (5 μg/ml) at 37 °C for 20 min and rinsed twice with PBS. Mitochondrial membrane potentials were supervised by determining the relative amounts or dual emissions from both mitochondrial JC-1 monomers and aggregates using an Olympus fluorescent microscope under Argon-ion 488 nm laser excitation. Mitochondrial depolarization was demonstrated by an increase in the ratio of green/red fluorescence intensity.

2.6. Nuclear morphology determination

PASMCs cultured in six-well plates after indicated treatments for 24 h in hypoxia were washed with PBS twice and then stained with acridine orange (AO) in lucifugal conditions for 10 min. The AO-stained cells were imaged with a fluorescent microscope at 488 nm laser excitation and 405 nm emissions. For each well, 20–30 shot were randomly selected to confirm the percentage of apoptotic cells in total cells based on the morphological characteristics of apoptosis. Cells with nuclear crenation, condensation and fractionation were defined as apoptosis cells.

2.7. TUNEL

The TUNEL assays were performed with the one step TUNEL kit according to the manufacturer's instructions. PASMCs treated as indicated were fixed with 4% paraformaldehyde phosphate buffer saline, washed with PBS, and then permeabilized by 0.1% Triton X-100 for 2 min on ice followed by TUNEL for 1 h at 37 °C. The FITC-labeled TUNEL-positive cells were imaged under a fluorescent microscopy under 488 nm excitation and 530-nm emission. The cells with green fluorescence were defined as apoptotic cells.

2.8. RT-PCR

Total RNA was prepared from treated PASMCs by Trizol extraction and quantitated by ultraviolet spectrophotometry (absorbance at 260 nm/280 nm). Reverse transcription was performed with the Superscript first-strand cDNA synthesis kit (Invitrogen) and