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PGC-1α plays a major role in the anti-apoptotic effect of 15-HETE in pulmonary artery endothelial cells

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ABSTRACT

Peroxisome proliferator activated receptor gamma coactivator 1α (PGC-1α) has been confirmed as a key regulatory factor in pulmonary artery smooth muscle cells to mediate mitochondrial biogenesis and proliferation during hypoxia. However, the functional role of PGC-1α in hypoxic pulmonary artery endothelial cells (PAECs) still needs to be determined. In the present study, we found a marked elevation in the expression of PGC-1α under hypoxia, which was predominate in the nucleus of PAECs. This alteration of PGC-1α showed a significant association with 15-Hydroxyeicosatetraenoic acid (15-HETE), a regulator known to be protective against apoptosis at the concentration of 1 μM. By silencing PGC-1α, the action against cell viability suppression induced by 15-HETE was blocked, not only in normoxic condition but also in hypoxia-stimulated condition. Likewise, the tendency to reduce TUNEL-positive cells, abnormal nuclei and apoptotic cells in response to 15-HETE was depending on PGC-1α. Furthermore, 15-HETE and PGC-1α siRNA caused significant alterations in related mechanisms including caspase activity, mitochondrial membrane potential, and Bcl-2 expression. Taken together, these results provide the first evidence to confirm the importance of PGC-1α in mediating the protective effect of 15-HETE against apoptosis. Therefore, a clear role of PGC-1α in hypoxic PAECs is demonstrated, which may be attributed to pulmonary vascular remodeling.

1. Introduction

It is well known that pulmonary vascular remodeling (PVR) serves as a considerable pathogenesis in the development of pulmonary artery hypertension (PAH), leading to right ventricular overload, heart failure and death. Chronic hypoxia is the most common contributor to both clinical and experimental PVR (Pak et al., 2007; Vender, 1994). To date, numerous investigators have made efforts to identify the precise mechanisms of hypoxia induced PVR. It is documented that each cell type in the layers of vascular wall, such as endothelial, smooth muscle and fibroblast, shows significant resistance against apoptosis, and plays a crucial role in the thickening of intima, media and adventitia during PVR (Humbert et al., 2004).

We note that pulmonary artery endothelial cells (PAECs) obtained from idiopathic PAH patients have decreased apoptosis as compared with control cells, and the raised number of endothelial cells in pulmonary vessels and capillaries is inducible by chronic hypoxia (Howell et al., 2003; Masri et al., 2007). However, an in-depth understanding of suppressed apoptosis in PAECs under hypoxia, calls for more detailed knowledge of the cellular and molecular mechanisms underlying.

Peroxisome proliferator activated receptor gamma coactivator 1α (PGC-1α) is a transcriptional coactivator, which have been identified to activate various transcription factors, such as peroxisome proliferator activated receptor gamma, estrogen receptor-related α, insulin-sensitive glucose transporter, nuclear respiratory factor (Gleyzer et al., 2005; Michael et al., 2001; Puigserver et al., 1998; Schreiber et al., 2004). As a consequence, multiple functions including nuclear and mitochondrial gene expression, energy metabolism and oxidative stress could be modulated by PGC-1α. Besides, previous studies show that PGC-1α plays an important role in regulation of anti-apoptosis, with relation to increased mitochondrial activity, oxidative stress genes and fatty acid oxidation (Valle et al., 2005; Won et al., 2010).

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Recent findings have demonstrated elevated PGC-1α expression in PAH patients, and proved the necessity of PGC-1α in pulmonary artery smooth muscle cells during hypoxia (Mata et al., 2012; Rao et al., 2012). As there are few reports evaluating PGC-1α in hypoxic PAECs, we first pay attention to investigate this issue.

Abundant formation of 15-Hydroxyeicosatetraenoic acid (15-HETE) is catalyzed by two isoenzymes of 15-lipoxygenase (15-LO), 15-LO1 and 15-LO2 in hypoxic pulmonary vessels as compared with controls (Zhu et al., 2003). It has been clarified that apoptosis depression of smooth muscle cells is depending on 15-LO/15-HETE under hypoxia (Ma et al., 2011). Combined with the evidence that 15-HETE could trigger the interaction of peroxisome proliferator activated receptor gamma and PGC-1α in fibroblast (Naruhn et al., 2010), a question whether 15-HETE has beneficial effects on PGC-1α in PAECs is raised.

Based on the information above, we proposed a hypothesis that PGC-1α might participate in the protective effect against apoptosis in PAECs under hypoxia exposure, and this process might be modulated by 15-HETE. In the current research, we specifically silenced PGC-1α expression and evaluated a series of anti-apoptotic events of 15-HETE.

2. Materials and methods

2.1. Materials

15-HETE and cinnamyl 3,4-dihydroxy-[alpha]-cyanocinnamate (CDC) were obtained from Cayman Chemical (Ann Arbor, Michigan, USA). Antibodies against PGC-1α and caspase-9 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Antibodies against Bcl-2, β-actin, and 4′,6-diamidino-2-phenylindole (DAPI), the TUNEL kit, annexin V-FITC apoptosis detection kit as well as JC-1 probe were from Beyotime Institute of Biotechnology (Haimen, China). Enhanced chemiluminescence agents (ECL) were obtained from Amersham-International (Amersham, UK). All other reagents were from common commercial sources.

2.2. Cell culture

Primary PAECs were prepared with branches of the main pulmonary artery (third or fourth division) of neonatal bovines obtained from an authorized slaughterhouse, according to the procedure we previously reported (Ma et al., 2011). In brief, the arteries were slit open, and cells were harvested by gentle scraping along the intimal surface of vessels with a surgical blade. All protocols were approved by the Ethical Committee of Laboratory Animals at Harbin Medical University. The purity of PAECs in the primary cultures had been confirmed by immunocytochemistry with antibody against CD31, and cells were cultured with 20% fetal bovine serum (FBS)-DMEM in a 37 °C, 5% CO_2 humidified incubator. Passages 3–6 were used for further experiments.

2.3. Western blot analysis

Proteins were extracted as we described previously (Ma et al., 2011). The concentration was examined by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). After electrophoresed on 8–12% SDS-PAGE, proteins were transferred onto the nitrocellulose membranes, and incubated in a blocking buffer (Tris 20 mM, pH 7.6, NaCl 150 mM, and Tween20 0.1%) containing 5% nonfat milk for 1 h. Followed by conjugation with primary antibodies (PGC-1α 1:200, procaspase-9 and caspase-9 1:200, Bcl-2 1:800, β-actin 1:5000) at 4 °C overnight, horseradish peroxidase-conjugated secondary antibodies were incubated for 1 h. Blots were developed using ECL reagents.

2.4. Immunocytochemistry and nuclear morphology determination

PAECs were cultured on a cover glass (15 mm diameter). After exposed to hypoxia (3% O_2) or 15-HETE (1 μmol/L) for 24 h, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 3% normal bovine serum. For immunocytochemistry, antibody specific to PGC-1α (1:25) was incubated at 4 °C overnight, and FITC-conjugated secondary antibody diluted with PBS was added away from light. For nuclear morphology determination, this step was skipped. Following a reaction with DAPI for 5 min, positive staining were detected and recorded by a fluorescence microscope (Nikon).

2.5. Small interfering RNA (siRNA) transfection

To knockdown the expression of 15-LO1/2 and PGC-1α, the siRNA designed and synthesized by GenePharma was transfected. Non-targeted control siRNA (CTL siRNA) was used as negative control. The sequences of siRNA were listed below: 15-LO1 siRNA: (NM: 031010) 5′-CCACCAAGGAAGCAACAAUTT-3′, 15-LO2 siRNA: (NM: 153301) 5′-GACCCUAUUAUACCAGAGUTT-3′, PGC siRNA: (NM_174745.2) 5′-GCACGCAGUCCUAUUCAUUTT-3′, CTL siRNA: 5′-UUCUCCGAACGUGUCACGUTT-3′. siRNA were transfected with X-tremeGene siRNA Transfection Reagent (Roche Applied Science, Mannheim, Germany) according to manufacturer's instructions. The sequences of 15-LO1 siRNA and 15-LO2 siRNA were the same as we reported previously and the efficiency of 15-LO1/2 interference had been clearly confirmed before (Li et al., 2013). PGC-1α silencing was verified by Western blot in Fig. 3B.

2.6. MTT

PAECs were seeded in 96-well plates at a density of 5000 cells/well, and subjected to growth arrest for 24 h before experimental treatment. At the end of experimental treatment, cells were incubated with 0.5% MTT in DMEM at 37 °C for 4 h. The reaction was terminated by adding dimethyl sulfoxide. The absorbance was read at 490 nm by a spectrophotometer.

2.7. TUNEL

TUNEL assay was performed to label the fragmented DNA of apoptotic PAECs. Prepared cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with TUNEL reagent for 1 h at 37 °C in the dark. The FITC-labeled TUNEL-positive cells with green fluorescence were imaged under a fluorescence microscope (Nikon) and indicated as apoptotic cells.

2.8. Flow cytometry

Annexin V-FITC apoptosis detection kit was applied to determine the ratios of apoptotic cells. Briefly, after exposed to the different experimental conditions, cells were collected by centrifugation and washed with cold PBS buffer. Then the cells were resuspended in 100 μL binding buffer, with 5 μL Annexin V-FITC and 10 μL PI in it, for 15 min at room temperature in the dark. The percentages of apoptotic cells were analyzed by a flow-cytometer.

2.9. Mitochondrial membrane potential assay

Mitochondrial depolarization was measured with JC-1 probe. First of all, prepared cells in 6-well plates were incubated with JC-1