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15-HETE protects pulmonary artery smooth muscle cells against apoptosis via SIRT1 regulation during hypoxia

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ABSTRACT

15-Hydroxyeicosatetraenoic acid (15-HETE) is produced by the catalytic metabolism of arachidonic acid by the enzyme 15-lipoxygenase. It is produced during hypoxia, and participates in the remodeling of pulmonary artery smooth muscle (PASM). Previous research has revealed that sirtuin 1 (SIRT1) involved in apoptosis in various cells and tissues. Herein, we attempted to determine whether 15-HETE counteracts SIRT1-promoted cell death in murine PASM cells (PASMCs). To verify this theory, we investigated changes in SIRT1 concentration in response to the counteraction of cell death by 15-HETE. We used western blotting and a terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay, and investigated the survival, nuclear morphology, and mitochondrial potential of the cells. Our results revealed that 15-HETE promotes the transcription and translation of SIRT1. Moreover, 15-HETE increases viability and impaired mitochondrial depolarization, and promotes the expression of Bcl-2 and Bcl-xL in PASMCs without serum. The reactions mentioned above were eliminated by SIRT1 inhibitors (EX 527 and SIRT1 inhibitor IV). Our findings suggest that 15-HETE is crucial for the protection of PASMCs against cell death, and the SIRT1 pathway may provide a new strategy for pulmonary artery hypertension therapy.

1. Introduction

Pulmonary artery hypertension (PAH) often leads to heart failure and mortality [1–3]. Pulmonary vascular remodeling (PVR) features medial hypertrophy and narrowing of the lumina of the small pulmonary arteries (PAs) arising from increased numbers of pulmonary artery smooth muscle cells (PASMCs) [4,5]. Malfunctioning cell death and cell proliferation result in an increase in the number of PASMCs (5), which contributes to PVR [6]. Chronic low levels of oxygen bring about PAH and suppress cell death in various cells such as PASMCs via unknown pathways [7,8]. Elucidation of the mechanisms by which these processes occur could provide innovative strategies for the treatment of PAH.

Sirtuins (the name is derived from the homologous yeast gene “silent mating type information regulation 2″) are capable of deacetylating proteins at their lysine residues [9,10]. With regard to mammals, seven sirtuins (SIRT1–7) have been found in various subcellular compartments [11,12]. Sirtuins modulate the functions of various substrate proteins, thereby modulating transcription, metabolism, the stability of the genome, and viability [13,14]. Research has demonstrated the expression of constitutive SIRT1 in the epithelia of large airways, and in smooth muscle cells (SMCs), macrophages, and the endothelial cells of vessels [15–17]. SIRT1 expression is stimulated in multiple cells in response to treatment with lipopolysaccharides, chronic hypoxia, proinflammatory cytokines, sepsis, or septic shock [18,19].

This study aimed to determine whether 15-hydroxyeicosatetraenoic acid (15-HETE) counteracts SIRT1-promoted cell death in murine PASM cells (PASMCs).

2. Materials and methods

2.1. Animals

We used adult male Sprague-Dawley (SD) rats (4–6 weeks) (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China). The animal study protocol was approved by the Committee on the Ethics of Animal Experiments of Deparment of Physiology of Harbin Medical University.

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2.2. Cell culture and reagents

Primary cultivated PASMCs were generated from tissues obtained from rats (4–6 weeks). The dissociated cells were centrifuged, and then resuspended in DMEM with 20% fetal bovine serum (FBS) in a 37°C, 5% CO_2 humidified incubator.

15-HETE was provided by Cayman Chemical (Ann Arbor, MI, USA), and was dissolved in ethanol before preservation in a nitrogen freezer. The SIRT1 inhibitors (EX 527 and SIRT1 inhibitor IV) were purchased from Santa Cruz Biotechnology.

2.3. Western blotting (WB)

Protein concentrations were determined with the BCA Protein Quantitation Kit (Genscript, Piscataway, USA). Proteins were separated using 10% SDS-PAGE and blotted electrophoretically onto polyvinylidene difluoride membranes. Antibodies against SIRT1, Bcl-2, cleaved caspase-3, Bcl-xL, and β-actin (Santa Cruz Biotechnology, Dallas, USA) were used in this study. Horseradish peroxidase-linked secondary antibodies (Santa Cruz Biotechnology) were incubated with the membranes for 1 h and followed by chemiluminescent detection.

2.4. Small interfering RNA (siRNA) design and transfection

We cultivated the PASMCs until they reached 30–50% confluence. Incubation was carried out in DMEM without serum to simulate starvation. siRNA (200 pmole) was independently dissolved in Opti-MEM-1 medium without serum and mixed together. The admixture (siRNA/transfection reagent) was incubated for 20 min at room temperature and was used to directly supplement the cells. The transfection reagent was removed 6–8 h after siRNA treatment. WB was carried out to evaluate the efficiency of protein silencing.

2.5. qRT-PCR

RNA was isolated from the cultivated PASMCs. A complementary DNA (cDNA) synthesis kit was used for reverse transcription. The cDNA was amplified using a DNA thermal cycler (PerkinElmer, Bridgeville, PA, USA). β-Actin served as an internal reference during PCR quantification.

2.6. MTS assay

The PASMCs were placed in 96-well plates (1×10^4 cells/well). The wells were supplemented with a series of levels of idelalisib, and incubated for 72 h. A MTS assay kit was obtained from Promega (Fitchburg, WI, USA).

2.7. Apoptosis

We cultivated the PASMCs in 6-well plates for 24 h using basal media. Subsequently, we added one of: SIRT1 inhibitor IV; EX 527; 15-HETE; 15-HETE plus SIRT1 inhibitor IV; or 15-HETE plus EX 527. Apoptosis was determined by Hoechst 33,258 (Invitrogen) and Annexin V/propidium iodide (PI) staining.

2.8. Evaluation of caspase-3 activity

Caspase-3 activity was assessed by investigating the cleavage of the chromogenic caspase-3 substrate. We determined absorbance at 405 nm, and OD_{405} served as an indication for the amount of caspase-3. Proteins were subjected to WB. Incubation was carried out for 2 h at 37 °C. We used a spectrometer to determine the absorbance at 405 nm of the yellow pNA that had been cleaved from the precursor. The activity of caspase-3 was normalized to the total proteins of the cell lysates.

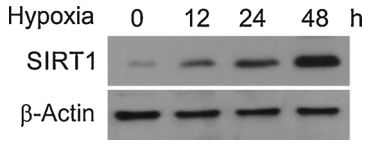

Fig. 1. SIRT1 upregulation during hypoxia. PASMCs were subjected to hypoxia for various periods to investigate the changes in SIRT1 protein expression.

2.9. Statistical analysis

The results were presented as means ± SD. The data was analyzed by Student *t*-test or a one-way analysis of variance (ANOVA) by using Prism version 5. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. SIRT1 upregulation during hypoxia

WB was used to verify the influence of hypoxia on SIRT1 translation. We discovered that low levels of oxygen (2.5% O_2/5% CO_2/balance N_2) triggered an obvious time-dependent upregulation of SIRT1 protein levels (Fig. 1).

3.2. 15-HETE promoted the transcription and translation of SIRT1 in the cultured PASMCs

WB and qRT-PCR were used to determine the influence of endogenous and exogenous 15-HETE on the expression of SIRT1 during normoxia and hypoxia. We found that hypoxia promoted the transcription and translation of SIRT1 compared with normoxia (Fig. 2A and B). The level of protein and mRNA were promoted if the cells received treatment with 15-HETE (Fig. 2A–D). However, the addition of nordihydroguaiaretic acid (NDGA), which is a selective arachidonate 15-lipoxygenase (15-LO) inhibitor, significantly eliminated the influence of 15-HETE (Fig. 2A–D). With regard to independent research, suppressing the production of 15-HETE using siRNA, or using NDGA to achieve 15-LO-2 knockdown, attenuates SIRT1 upregulation triggered by hypoxia, indicating that endogenous 15-HETE regulates SIRT1 expression triggered by hypoxia (Fig. 2E and F).

3.3. 15-HETE promoted PASMC survival via SIRT1 pathway

MTS assay was used to evaluate the influence of 5-HETE on PASMC survival. During normoxia and hypoxia, serum deprivation brought about a noticeable suppression of PASMC survival. However, 15-HETE defended survival following serum deprivation, which was markedly impaired by 2 μM EX 527 or 2 μM SIRT1 inhibitor IV during normoxia and hypoxia (Fig. 3A and B).

3.4. 15-HETE stimulated the expression of Bcl-xL and Bcl-2 via SIRT1 pathway

Bcl-xL and Bcl-2 prevent cell death. They were discovered on the membranes of mitochondria, and are related to mitochondrial activity, participating in cell death. Bcl-xL and Bcl-2 expression was promoted by 15-HETE. However, EX 527 and SIRT1 inhibitor IV partly impaired their influence (Fig. 4). Our research suggests that 15-HETE modulates mitochondrial membrane proteins that prevent cell death partially via an SIRT1 pathway, thereby preserving the mitochondria.

3.5. 15-HETE suppressed the stimulation of caspase-3 activation

Caspase-3 is generated from a precursor named procaspase-3, which