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Protective effect of sphingosine-1-phosphate for chronic intermittent hypoxia-induced endothelial cell injury

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ABSTRACT

Intermittent hypoxia (IH) induced by obstructive sleep apnea (OSA) is the key factor in oxidative stress and the concomitant inflammation of endothelial cells (ECs). In recent years, the lipid sphingosine-1-phosphate (S1P) has been reported to probably play a central role in inflammatory diseases. However, its role in IH-induced endothelial injury remains uncertain. In this study, we investigated the IH-induced ECs inflammation and apoptosis, as well as the role of S1P in both. First, human umbilical vein endothelial cells (HUVECs) were treated with IH to explore the mechanism of S1P and S1P microbubbles (S1P-MBs) in HUVECs with altered function. The intracellular reactive oxygen species (ROS) significantly increased after IH treatment, which further resulted in the increased efficiency of cell apoptosis. Following the S1P and S1P-MBs treatments, the lower Bax protein and Cyt c protein levels in HUVECs indicated the protective effects of S1P for CIH-induced ECs injury. The reason may be that the enhanced expression levels of $G\alpha_{(i)}$ and S1P receptor 1 in S1P and S1P-MBs treatment groups could actively increase intracellular p-Akt and p-eNOS protein levels, which counteract the increased ROS secondary to inflammation from IH. Therefore, the Akt/eNOS signaling pathway induced by S1P may be important in protecting IH-induced ECs injury. Furthermore, the S1P-MBs may be designed as a novel S1P dosage formulation to protect the body from the ECs injuries in the future.

1. Introduction

Obstructive sleep apnea (OSA), which is characterized by IH, is a highly prevalent breathing disorder during sleep. Moreover, blood hypoxemia, hypercapnia, sleep fragmentation, augmented respiratory efforts, and increased sympathetic activity are affected by OSA [1]. Studies have shown OSA to be an independent risk factor for cardiovascular morbidity [2]. Additionally, data from clinical and experimental studies have suggested that IH is the main component that links OSA to atherosclerosis [3]. Therefore, oxidative stress and concomitant inflammation are two highly prominent underlying mechanisms that may explain the association of OSA to atherosclerosis.

Sphingosine-1-phosphate (S1P), which is produced by the ATP-dependent phosphorylation of sphingosine and catalyzed by two sphingosine kinases, namely, SPHK1 and SPHK2, is a pleiotropic lipid with various physiological and pathophysiological roles [4]. The S1P can function as an intracellular second messenger to induce cellular responses, including proliferation, migration, contraction, and intracellular calcium mobilization. Currently, many S1P actions are mediated through the subtypes of S1P G protein-coupled receptors, which comprise $S1PR_{1\text{-}5}$. Moreover, ECs express $S1PR_1$, $S1PR_2$, and $S1PR_3$. The $S1PR_1$ is the most abundant subtype, whereas others are expressed at lower levels [5]. S1P-mediated responses, such as migration, angiogenesis, and adherent junction formation, on ECs mainly occur *via* the G_i-mediated activity of $S1PR_1$ [6]. Furthermore, $S1PR_1$ is an anti-inflammatory receptor, according to growing evidence. The stimulation of $S1PR_1$ reduces the adhesion of monocytes to ECs [7]. Therefore, the S1P-related molecular basis of ECs injury is important in the development of novel anti-inflammatory therapeutics.

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Microbubbles (MBs) are gas-filled structures that are stabilized by a lipid, protein, or polymer shell; some MB types have been clinically approved as ultrasound contrast agents due to their gas-filled and compressible cores [8]. Recently, as acoustic-response drug delivery systems, MBs have been developed as novel drug carrier formulations to deliver drugs, peptides, and genes [9]. In this study, we aimed to prove that IH-caused ECs injury may benefit from the addition of S1P molecules. We prepared S1P-MBs and results showed the same function of improving ECs injury compared with S1P. Therefore, S1P and S1P-MBs have protective roles for CIH-induced ECs injury. Furthermore, the increased p-Akt and p-eNOS protein levels for S1P and S1P-MBs treatment groups demonstrate that the addition of S1P molecules could actively increase the expression levels of $S1PR_1$ and $G\alpha_{(i)}$. The Akt/eNOS signaling pathway induced by S1P may be crucial in protecting from ECs injury. Furthermore, S1P might have a therapeutic potential for treating IH-induced ECs injury.

2. Materials and methods

2.1. Materials

Human umbilical vein endothelial cells (HUVECs) were purchased from Sciencell (Cat No.8000) and cultured in endothelial cell medium (ECM, Cat No.1001, Sciencell). S1P (S9666, Sigma) from methanol stocks was air-dried and dissolved in phosphate-buffered saline/1% bovine serum albumin. Afterward, S1P was diluted in ECM. Moreover, 1, 2 distearoyl-*sn*-glycero-3-dipalmitoylphosphatidylcholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA), 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamin-*N*-[(polyethylene glycol)-5000] (DPPE-PEG5000), all in powder form, were purchased from Avanti Polar Lipids Inc. (Alabaster, USA). $S1PR_1$ antagonist (W146) was purchased from Tocris (UK). PI3 Kinase Inhibitor (LY294002), antibodies against eNOS, p-eNOS, Akt, p-Akt, $G\alpha_{(i)}$, Bax, Bcl2, Cyt c, and cleaved-caspase 3 were purchased from Cell Signaling Technology (USA). Anti-$S1PR_1$ antibody was purchased from Sigma. TUNEL apoptosis Detection Kit was purchased from Roche (Germany). Reactive oxygen species (ROS) Detection Kit was purchased from Beyotime (China).

2.2. IH exposure and cell culture

The normal HUVECs were cultured in ECM supplemented with 5% FBS and 1% EC growth supplement. The IH-induced injured HUVEC cells were incubated at 37 °C at 5% CO_2 in a chamber (PUHE Biotechnology Company LTD, China), in which O_2 levels were alternated between 21% for 25 min and 1% for 35 min, for a total of 72 cycles (72 h). The medium was changed every 48 h. Cells in the control group were maintained under normoxic conditions (IN: 21% O_2 and 5% CO_2) throughout the study.

According to the experimental protocol, the cells were stimulated with PBS, S1P, and MBs with and without S1P.

2.3. The preparation of S1P-MBs

Appropriate amounts of DPPC (25 mg/ml, 53 μl), DPPA (25 mg/ml, 6 μl), and DPPE-PEG5000 (25 mg/ml, 40 μl) in chloroform were mixed. The S1P chloroform solution (1 mg/ml, 190 μl) was uniformly mixed with the above lipid solution. Chloroform was removed, followed by evaporation under a vacuum and then mild sonication with a bath sonicator (40 kHz, 130 W, 10 min). The liposome solution was aliquoted in 1 ml lots to a 3-mL vial with decafluorobutane gas in the headspace. According to the above formulation, the final concentration of lipid was 2.5 mg/ml and the S1P concentration was 0.19 mg/ml. The vial underwent mechanical agitation through a VialMix shaker (Mixtura, ImaRx Therapeutics, Tucson, AZ) for 60 s. Then wash 3 times using 10 mM PBS to remove the submicro-sized bubbles and remaining liposomes by flotation at 300×*g* for 3 min to obtain S1P-MBs. The control MBs were prepared with the same methods, but without adding the S1P.

To analyze the effects of S1P-MBs without ultrasound (US), the HUVECs were seeded in four culture dishes: negative control group (NC), normal cells with S1P-MBs group, IH group, IH + S1P-MBs group. The cells were further incubated for 72 h in normal culture incubator or IH culture incubator. The protein levels of Bcl2, Bax, and Cyt c were tested by Western blot analysis.

2.4. Assay effects of S1P and S1P-MBs on the ECs dysfunction

For the S1P treatment experiment, the HUVECs were divided into four groups: NC group, IH group, IH + PBS group and IH + S1P group. The cells were further incubated for 72 h in normal culture incubator or IH culture incubator. The levels of ROS, apoptosis, $S1PR_1$, $G\alpha_{(i)}$, and other proteins were assessed according to the manufacturer's protocols.

For the S1P-MB treatment experiment, HUVEC cells were divided into various treatment groups: (a) normal cells with US (NC + US); (b) normal cells incubated with normal MBs under the exposure of US (NC + MBs + US); (c) normal cells incubated with S1P-MBs followed by US (NC + S1P-MBs + US); (d) IH-induced cells with US (IH + US); (e) IH-induced cells incubated with normal MBs under the exposure of US (IH + MBs + US); and (f) IH-induced cells incubated with S1P-MBs followed by US (IH + S1P-MBs + US). Different samples were added in the culture plates according to the different terms. The ultrasonic probe was vertically placed about 8 cm from the top of the culture plates and was continuously irradiated for 40 s (1 MHz, 0.5 W/cm^2). After ultrasonic intervention, the cells were further incubated for 72 h in normal culture incubator or IH culture incubator. The ROS level and apoptosis were assessed according to the following protocols.

To observe the differences between S1P and S1P-MBs on ECs dysfunction and verify the protection of S1P by inducing the S1PR1/Akt pathway, cells were divided into eight groups: NC group, IH group, IH + LY294002 group (IH + PI3K inhibitor), IH + LY294002 + S1P group, IH + LY294002 + S1P-MBs with US group, IH + W146 group (IH + S1PR1 antagonist), IH + W146 + S1P group, and IH + W146 + S1P-MBs with US group. The protein levels of p-eNOS/eNOS, p-Akt/Akt, S1PR1, $G\alpha_{(i)}$, Bcl2, Bax, Cyt c were assessed according to the following protocols.

S1P was administered at 100 nmol/L in both S1P and S1P-MB treatment groups.

2.5. Immunofluorescence microscopy and apoptosis detection

Immunofluorescence was performed as described [10]. After fixation and blocking, cells were incubated with primary antibody against $S1PR_1$ (1:100) and $G\alpha_{(i)}$ (1:100) overnight at 4 °C, then washed with PBS and incubated with secondary antibody for 30 min at 37 °C (Alexa Fluor 488 and 549 goat anti-rabbit; Alexa Fluor 549 and 488 goat anti-mouse 1:200). The nuclei were counterstained with DAPI (Beyotime, China). Specific fluorescent images were acquired by using a fluorescence microscope (IX-71, Olympus, Japan).

Intracellular ROS was detected with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime) following the manufacturer's instructions. The apoptotic cells were detected using an in situ cell death detection POD Kit (Roche, Germany) following the manufacturer's instructions. and finally analyzed the samples under a light microscope.