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Original article

Salidroside attenuates chronic hypoxia-induced pulmonary hypertension via adenosine A_{2a} receptor related mitochondria-dependent apoptosis pathway

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ABSTRACT

Pulmonary arterial hypertension (PAH) is characterized by pulmonary arterial remodeling mainly due to excess cellular proliferation and apoptosis resistance of pulmonary arterial smooth muscle cells (PASMCs). Salidroside, an active ingredient isolated from Rhodiola rosea is proposed to exert protective effects against PAH. However, the function of salidroside in PAH has not been investigated systematically and the underlying mechanisms are not clear. To investigate the effects of salidroside on PAH, the mice in chronic hypoxia model of PAH were given by an increasing concentration of salidroside (0, 16 mg/kg, 32 mg/kg, and 64 mg/kg). After salidroside treatment, the chronic hypoxia-induced right ventricular hypertrophy and pulmonary arterial remodeling were attenuated, suggesting a protective role played by salidroside in PAH. To explore the potential mechanisms, the apoptosis of PASMCs after salidroside treatment under hypoxia conditions were determined in vivo and in vitro, and also the mitochondria-dependent apoptosis factors, Bax, Bcl-2, cytochrome C, and caspase 9 were examined. The results revealed that salidroside reversed hypoxia-induced cell apoptosis resistance at least partially via a mitochondria-dependent pathway. In addition, salidroside upregulated the expression of adenosine A_{2a} receptor ($A_{2a}R$) in lung tissues of mice and in PASMCs in vitro after hypoxia exposure. Combined the evidence above, we conclude that salidroside can attenuate chronic hypoxia-induced PAH by promoting PASMCs apoptosis via an $A_{2a}R$ related mitochondria dependent pathway.

1. Introduction

Pulmonary arterial hypertension (PAH) is a subset of pulmonary hypertensive syndromes and characterized by pulmonary arterial remodeling, leading to increased pulmonary vascular resistance and increased pulmonary arterial pressure [1–3]. PAH is a debilitating disease and results ultimately in right ventricular failure and death. It is reported that PAH appears approximately in 12–50 per million in adult people [4]. Chronic hypoxia-induced PAH is a common type of PAH, mainly secondary to disorders of the respiratory system, such as chronic obstructive pulmonary disease (COPD), obstructive sleep apnea, interstitial lung disease, and chronic mountain sickness in plateau residents. Pulmonary arterial remodeling is mainly due to the abnormal growth, the excess cellular proliferation, and the apoptosis resistance of smooth muscle cells [5–7]. Therefore, inhibition of the cell proliferation or induction of the cell apoptosis may be an efficient therapeutic strategy for PAH.

Mitochondrial dysfunction and mitochondrial-dependent apoptotic pathway has been identified to be involved in the pathogenesis of pulmonary arterial hypertension and play a critical role in hypoxic apoptosis of PASMCs [8–12]. The mitochondria dependent pathway starts with an apoptosis-regulating protein family exemplified by Bcl-2 family, such as pro-apoptotic Bax and anti-apoptotic Bcl-2 [13–15]. Pro-apoptotic factor, Bax, can enhance cytochrome C release from mitochondria [13,14]. And cytochrome C release into cytosol activates caspase 9 which then activates caspase 3 to execute the apoptotic program [13,15]. Whereas, the anti-apoptotic molecule, Bcl-2, can inhibit the activation of Bax and the release of cytochrome C, consequently inhibits cell apoptosis [15]. Previous researches reveal that the increased Bax/Bcl-2 ratio can reflect the activation of caspase program and the induction of apoptosis [16]. Mitochondria dependent apoptosis is suppressed in hypoxic human PASMCs (HPASMCs) in vitro and in PASMCs from patients and rats with PAH [17].

Abbreviations: PAH, pulmonary arterial hypertension; PASMCs, pulmonary arterial smooth muscle cells; $A_{2a}R$, adenosine A_{2a} receptor; COPD, chronic obstructive pulmonary disease; HUVECs, human umbilical vein endothelial cells; MYH11, smooth muscle myosin heavy chain 11; RVSP, right ventricular systolic pressure; mCAP, mean carotid arterial pressure; VDCA, voltage-dependent anion channel.

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Salidroside is an active ingredient isolated from Rhodiola rosea, a member of Crassulaceae and a well-known herb used to relieve high altitude sickness and acute exacerbation of PAH [18]. Several experimental and clinical studies of salidroside have provide evidences for its multiple pharmacological activities including anti-inflammation [19], anti-oxidation [20], anti-stress, anti-cancer, and enhancing immune effects [18,21,22]. Recent studies indicate that salidroside can rescue mitochondria dysfunction induced by stimuli in human umbilical vein endothelial cells (HUVECs) [23], and also salidroside can block platelet-derived growth factor-BB induced proliferation of PASMCs [24], suggesting that salidroside may exert protective effects against PAH via rebalancing cell proliferation and apoptosis of pulmonary artery cells. However, the function of salidroside in PAH has not been investigated systematically and the underlying mechanisms are not clear.

In the present study, we explored the effects of salidroside on PAH and pulmonary arterial remodeling with a mouse model of chronic hypoxia-induced PAH and examined whether salidroside affects hypoxia-induced cell apoptosis resistance both in vivo and in vitro. In addition, the expression of adenosine A_{2a} receptor was also determined to verify the involvement of the receptor.

2. Materials and methods

2.1. Materials

Salidroside, A_{2a}R agonist CGS-21680, collagenase type I, and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were obtained from Sigma (St Louis, MO, USA). Trizol, fetal bovine serums (FBS), penicillin G, streptomycin, and Dulbecco's Modified Eagle Medium (DMEM, high glucose) were obtained from Life Technologies (CA, USA). Sso Advanced SYBR Green Supermix was obtained from Bio-Rad (Hercules, CA, USA). The rabbit antibodies against A_{2a}R, Bax, smooth muscle myosin heavy chain 11 (MYH11), and α-smooth muscle actin (SMA) were purchased from Abcam (Cambridge, UK). The rabbit antibodies against GAPDH, cytochrome C, cleaved caspase 3, cleaved caspase 9, voltage-dependent anion channel (VDCA, porin), and Bcl-2 were purchased from Cell Signaling Technology (MA, USA). The goat antibodies against Bax and Bcl-2 were purchased from Santa Cruz (CA, USA). Cell counting kit-8 (CCK-8) and TIANScript first strand cDNA synthesis kit were purchased from Dojindo Laboratories (Kumamoto, Japan) and TIANGEN BIOTECH (Beijing, China), respectively. SuperSignal (R) West Femto Maximum Sensitivity Substrate and BCA Protein Assay kit were purchased from Pierce (WI, USA). DAB kit and Polink-2 plus Polymer HRP Detection System were purchased from ZSGB BIO (Beijing, China). DyLight™ 488 Conjugated Goat anti-Mouse IgG (H + L) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Peroxidase conjugated goat anti-rabbit immunoglobulin G (IgG) was obtained from Jackson ImmunoResearch Laboratories (PA, USA). Alexa Fluor 647 Donkey anti-Goat IgG (H + L) Antibody, 555 Donkey Anti-Goat IgG (H + L), and 488 Donkey Anti-Rabbit IgG (H + L) Antibody were purchased from Molecular Probes (Eugene, OR, USA).

2.2. Experimental animals and chronic hypoxia model of PAH

Forty male BALB/C mice (12–14 week old, weight 22–25 g) were purchased from Silaike Experimental Animal Technology (Shanghai, China). Animal housing and experimental protocols were approved by Wenzhou Medical University. The mice were given free access to food and water and were maintained in a room with a 12:12 hour light–dark cycle between 20 and 24 °C. The mice were randomly assigned to five groups: normoxia group (N) ($n = 8$), hypoxia group (H) ($n = 8$), hypoxia plus salidroside groups (16 mg/kg, 32 mg/kg and 64 mg/kg, named by HS16, HS32, HS64, respectively) ($n = 8$). Intraperitoneal injection was given half an hour before mice were put in hypoxia chamber. The normoxia group was injected saline and exposed to room air whereas the hypoxia group was exposed to 9%–11% O_2 with the same injection. The hypoxia environment was established by a mixture of room air and nitrogen in a closed chamber. The fractional concentration of O_2 was monitored and controlled by a detector automatically. The hypoxia exposure lasted for four weeks.

2.3. Measurements of RV hypertrophy

At the end of the hypoxia exposure period, the mice were anesthetized with 20% urethane (1 mL/100 g, intraperitoneal (i.p.)). Two home-made polyethylene (PE) catheters, prefilled with heparin, were connected to the pressure transducers (PowerLab 8/35 multi-channel biological signal recording system, AD Instruments, Colorado Springs, CO, Australia) and inserted into the right ventricle and left carotid artery, respectively, and then the right ventricular systolic pressure (RVSP) and the mean carotid arterial pressure (mCAP) were recorded. Next, the hearts were dissected out, divided into right ventricle (RV), left ventricle (LV), and septum (S), and weighted, respectively. The weight ratio of RV to LV plus S and the weight ratio of RV to body were calculated as indexes to reflect RV hypertrophy.

2.4. Detection of pulmonary arterial remodeling

The upper lobe of right lung was fixed in 4% paraformaldehyde overnight, de-hydrated in increasing concentrations of ethanol, delipidated with xylene, embedded in paraffin, and sectioned at 5 μm thick. After hematoxylin and eosin (HE) staining, elastic fiber staining, and immunostaining with anti-α-SMA antibody, the structure remodeling of the pulmonary arteries was characterized by microscopic evaluation. The pulmonary arteries (external diameters of 25–100 μm) were chosen randomly at a magnification of 400× and analyzed with Image-Pro Plus, Version 6.0 (Media Cybernetics, USA). The part of α-SMA-staining positive represented the medial layer. The percentage of pulmonary artery wall thickness to total thickness (WA/TA%), the percentage of wall areas to total areas (WA/TA%), and the density of nuclei in the medial SMCs were calculated to evaluate pulmonary arterial remodeling.

2.5. Ultrastructural examination of pulmonary arteries

The lung tissues closed to the lung hilus was sectioned into small pieces (approximately $1 \times 1 \times 3$ mm^3), fixed with 2.5% glutaraldehyde and 1% osmic acid, dehydrated with acetone, and embedded in epoxy resin 812. And then the fixed tissues were cut into ultrathin sections with ultramicrotome and examined by a Hitachi H-7500 transmission electron microscopy (Hitachi, Japan) after staining with uranyl acetate and lead citrate. Five fields were obtained randomly.

2.6. Isolation and cell culture of PASMCs

Male BALB/c mice were killed by intraperitoneal injection of 10% chloral hydrate followed by cervical dislocation that was approved by the guideline of Wenzhou Medical College and National Institutes of Health Standards of Animal Care. The heart and lungs were removed, and the second to third branches of the intrapulmonary artery were dissected in sterile phosphate buffered saline (PBS). The pulmonary arterial tissues were digested by 0.2% collagenase type I and incubated for 40–80 min at 37 °C. Then the cells were cultured in glucose DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). The smooth muscle cell was identified by microscopy and positive staining for α-SMA.

2.7. Cell viability assay

A cell counting kit-8 (CCK-8, Dojindo, Japan) was used to detect the effect of salidroside on cell viability in PASMCs with hypoxia exposure or not. Mouse PASMC cells were seeded in 96 well plates (1×10^4 cells/well) and pretreated with increasing concentrations of salidroside