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Grape seed procyanidin extract attenuates hypoxic pulmonary hypertension by inhibiting oxidative stress and pulmonary arterial smooth muscle cells proliferation☆

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Abstract

Hypoxia-induced oxidative stress and excessive proliferation of pulmonary artery smooth muscle cells (PASMCs) play important roles in the pathological process of hypoxic pulmonary hypertension (HPH). Grape seed procyanidin extract (GSPE) possesses antioxidant properties and has beneficial effects on the cardiovascular system. However, the effect of GSPE on HPH remains unclear. In this study, adult Sprague–Dawley rats were exposed to intermittent chronic hypoxia for 4 weeks to mimic a severe HPH condition. Hemodynamic and pulmonary pathomorphology data showed that chronic hypoxia significantly increased right ventricular systolic pressures (RVSP), weight of the right ventricle/left ventricle plus septum (RV/LV+S) ratio and median width of pulmonary arteries. GSPE attenuated the elevation of RVSP, RV/LV+S, and reduced the pulmonary vascular structure remodeling. GSPE also increased the levels of SOD and reduced the levels of MDA in hypoxia-induced HPH model. In addition, GSPE suppressed Nox4 mRNA levels, ROS production and PASMCs proliferation. Meanwhile, increased expression of phospho-STAT3, cyclin D1, cyclin D3 and Ki67 in PASMCs caused by hypoxia was down-regulated by GSPE. These results suggested that GSPE might potentially prevent HPH *via* antioxidant and antiproliferative mechanisms.

Keywords: Grape seed procyanidin extract; Hypoxic pulmonary hypertension; Reactive oxygen species; Proliferation

1. Introduction

Hypoxic pulmonary hypertension (HPH) is a serious disease with significant disability and reduced life expectancy. Sustained hypoxia caused by primary lung diseases, including chronic obstructive pulmonary disease (COPD), cystic fibrosis, diffuse interstitial fibrosis, bronchopulmonary dysplasia, radiation fibrosis, infiltrative lung tumors and collagen vascular disease, leads to the development of increased pulmonary vascular resistance and pulmonary hypertension (PH) [1,2]. The elevation in pulmonary vascular resistance in HPH has been attributed to structural remodeling of the vessels, which lead to thickening of the walls of the pulmonary arteries and narrowing the vascular lumen [3]. The pulmonary vascular wall is made up of three resident cell types, the endothelial (intima), smooth muscle (media) and fibroblast (adventitia) cells [4]. Among them, the thickened media caused by hypoxia-induced excessive proliferation of pulmonary artery smooth muscle cells (PASMCs) is considered as hallmarks of hypoxia-induced pulmonary vascular remodeling and hypertension [5,6]. PASMCs are particularly sensitive to oxygen availability and responsible for acute hypoxic vasoconstriction and the development of PH due to chronic hypoxia [7]. Although HPH is very common, there is no specific treatment for this serious disease [8]; hunting for novel effective pharmacologic treatments for HPH is urgent.

Current evidence suggests that reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$) and hydroperoxyl radical ($HO_2^{\bullet}$) generated by mitochondria, NADPH oxidases (Noxes) and other enzymatic sources are recognized stimulus for vascular wall cell proliferation and vasoconstriction in PH pathogenesis [9,10]. In chronic hypoxia-induced PH, an increase in ROS production has been shown in the lung and pulmonary arteries [11]. A variety of compounds with antioxidant properties have been

Abbreviations: HPH, hypoxic pulmonary hypertension; PH, pulmonary hypertension; GSPE, grape seed procyanidin extract; PASMCs, pulmonary artery smooth muscle cells; ROS, reactive oxygen species; α-SMA, α-smooth muscle actin; PCNA, proliferating cell nuclear antigen; MDA, malondialdehyde; SOD, superoxide dismutase; RVSP, right ventricle systolic pressure; RV/LV+S, right ventricle/left ventricle plus septum; WT%, percent medial wall thickness; WA%, percent medial wall area.

☆ Conflicts and Interest: none.

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shown to significantly attenuate pulmonary vasoconstriction due to hypoxia and have beneficial therapeutic effects in PH [12]. Moreover, ROS serve as important intracellular and intercellular messengers to promote PASMCs proliferation and inhibit apoptosis [13]. Thus, targeting of ROS appears as a potential approach in PH treatment.

Grape seed procyanidin extract (GSPE), a biologically active polyphenolic flavonoid combination that contains oligomeric proanthocyanidin, has been reported to exert biological, pharmacological, therapeutic and chemoprotective properties against oxygen free radicals and oxidative stress [14,15]. Furthermore, GSPE provided significantly greater protection against damage of oxidative stress as compared to vitamins C, E and β-carotene [16]. In addition, it has been reported that GSPE has protective effects on various cardiac disorders, protect against structurally diverse drug and chemical-induced cardiotoxicity, correct dyslipidemia associated with high-fat diet, decrease arterial pressure and ameliorate atherosclerosis [16–20]. Although GSPE has been found to scavenge ROS and protect cardiovascular system, whether GSPE has effects on HPH has never been reported. The aim of this study was to investigate the possible effect and the underlying mechanisms of GSPE on HPH.

2. Materials and methods

2.1. Reagents and antibodies

GSPE was purchased from Tianjin Jianfeng Natural Product R&D Co., Ltd. (Tianjin, China). High-glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin solution, Trizol reagent, Alexa Fluor 488 goat antirabbit IgG conjugated antibody, Alexa Fluor 594 goat antimouse IgG conjugated antibody and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against phospho-STAT3 (p-STAT3, Tyr705), total STAT3, cyclin D1, cyclin D3, Ki67, GAPDH, horseradish peroxidase-conjugated goat antimouse and goat antirabbit were purchased from Cell Signaling (Beverly, MA, USA). Anti-α-SMA was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animal experiments

Twenty-eight male Sprague–Dawley rats (200–250 g) were obtained from the animal center of Dalian Medical University. All experiments were approved by the Institutional Animal Care and Use Committee of the Dalian Medical University. The rats were randomly divided into four groups (seven rats per group): (a) normoxia group; (b) normoxia group treated with GSPE; (c) hypoxia group; (d) hypoxia group treated with GSPE. For group (b) and (d), rats were administered GSPE (250 mg/kg/day), intragastrically dissolved in normal saline for 1 week before and for the entire 4 weeks of nomoxia or hypoxia exposure. The rats designated for exposure to chronic hypoxia were housed intermittently in a hypobaric hypoxia chamber for 10 h/d continuing 4 weeks. The hypoxic chamber was flushed with room air and 100% N_2 to maintain 10% O_2 concentration. The normoxic rats were housed at room air.

2.3. Hemodynamic experiments

After 4 weeks hypoxia exposure, the rats were anesthetized with 4.8% tribromoethanol (7.5 ml/kg *via* intraperitoneal injection), and a polyethylene catheter linked to a transducer was inserted through the right jugular vein into the right ventricle (RV). The right ventricle systolic pressure (RVSP) was then recorded using Power Lab Software (ADI Instruments). After that, blood samples, lungs and heart were obtained. The RV and left ventricle plus septum (LV+S) were collected, and the weight ratio of (RV/LV+S) was calculated as an index of RV hypertrophy. The lungs were dissected into 4-mm-thick slices and placed in 4% paraformaldehyde solution for 72 h. Blood samples were centrifuged, and serum was separated. Then the left lungs and serum were stored at −80 °C for subsequent experiments.

2.4. Morphological investigation

The lung slices were embedded in paraffin and cut into 4-μm thick sections and stained with hematoxylin and eosin as we previously reported [21]. Morphologic changes in the small pulmonary artery (50–200 μm) were detected using a Zeiss microscope digital camera. The outside diameter, inside diameter, medial wall area and total vessel area of pulmonary arteries were measured. The percent medial wall thickness (WT%) and percent medial wall area (WA%) were calculated to present pulmonary vascular structure remodeling. WT%=(outside diameter−inside diameter)/(outside diameter)×100; WA%=(medial wall area)/(total vessel area)×100.

2.5. Immunohistochemical staining

Sections were deparaffinized, rehydrated, retrieved the antigens and then incubated with 1% H_2O_2 in methanol for 15 min to block endogenous peroxidase. After blocked with 5% bovine serum albumin, sections were incubated overnight with anti-α-SMA mouse monoclonal antibody (1:500 dilution) at 4 °C. Then, a biotinylated antimouse IgG antibody and an avidin-biotinylated peroxidase complex were applied with 3,3-diaminobenzidine as a peroxidase substrate. Immunoreactivity was visualized using diaminobenzidine. Then a light hematoxylin counterstain was applied.

For the α-smooth muscle actin (α-SMA), a marker for smooth muscle cells (SMCs) to determine the expression of SMCs, quantitative immunohistochemical assessments were performed as previously reported [22].

2.6. Immunofluorescence staining

Double immunofluorescence staining for Ki67 and α-SMA was performed on sections. Sections were deparaffinized, rehydrated, retrieved the antigens and blocked endogenous peroxidase. After blocked with 5% bovine serum albumin, sections were incubated overnight with anti-α-SMA antibody (1:100 dilution), anti-Ki67 antibody (1:200 dilution) at 4 °C. Then, Alexa Fluor 488 (green) goat antirabbit IgG and Alexa Fluor 594 (red) goat antimouse IgG-conjugated antibodies were incubated on the sections for 1 h at room temperature. Cell nuclei were stained with DAPI. Images were taken by Olympus BX63 fluorescence and confocal microscopy.

2.7. Assay of MDA and SOD

The content of malondialdehyde (MDA) and superoxide dismutase (SOD) were measured using commercial kits (Beyotime Institute of Biotechnology Shanghai, China) and analyzed with a spectrophotometer. Detailed manipulation process was performed according to the manufacturer's instructions.

2.8. Primary cells culture and in vitro hypoxia

Primary PASMCs were cultured by tissue explant method [22] and grown in DMEM medium supplemented with 100-U/ml penicillin and 10% fetal bovine serum. PASMCs were identified by immunocytochemical staining for α-SMA at each passage. Cells were used for experiments between passages 3 and 6. For all experiments, cells were divided into six groups: normoxia, hypoxia, hypoxia+20-μg/ml GSPE, hypoxia+40-μg/ml GSPE, hypoxia+80-μg/ml GSPE and hypoxia+120-μg/ml GSPE. Cells were cultured either in 21% oxygen or 3% oxygen condition.

2.9. Detection of intracellular ROS

The level of intracellular ROS was measured using the Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology Shanghai, China) according to the manufacturer's instructions. DCFH-DA diffuses into cells and is hydrolyzed into nonfluorescent DCFH. Produced ROS oxidize nonfluorescent intracellular DCFH to highly fluorescent DCF. After cultured for 12 h, cells were collected and exposed to serum-free DMEM containing 10-μM DCFH-DA. After 20 min of incubation in the darkness, cells were washed with serum-free DMEM for three times, and then fluorescent intensity was measured by the flow cytometer on FL-1 channel with excitation and emission wavelengths of 488 and 525 nm, respectively. The data were recorded with the use of Flowing Software 2.0 as the “M2 percentage” fluorescence variation, which indicates the percentage of cells with enhanced ROS production.

2.10. Cell proliferation analysis

The effect on proliferation of GSPE was analyzed using the Trypan Blue dye-exclusion assay. The cells were seeded at a density of 2×10^4 cells per well in a 24-well culture plate. After cultured for 48 h, the cells were harvested with trypsinization. Trypan Blue (0.4%) was added, and the number of viable cells that excluded the dye was counted with a hemocytometer.

2.11. Western blotting analysis

After cultured for 12 h, cells were lysed in a protein extraction buffer containing 50-mM Tris (pH 7.4), 150-mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2-mM NaF, 5-mM EDTA (pH 8.0), 1-mM sodium orthovanadate. The protease inhibitor of phenylmethylsulfonyl fluoride (PMSF, 1 mM) was added to the buffer in advance. The samples were separated on 10% SDS-polyacrylamide gels and then transferred to a nitrocellulose membrane. The primary antibodies were p-STAT3 antibody (1:1000), total STAT3 antibody (1:2000), cyclin D1 antibody (1:1000) and cyclin D3 antibody (1:2000). The signals were detected by ECL kit (Amersham Biosciences, Little Chalfont, UK).

2.12. Quantitative real-time RT-PCR analysis

Cells were cultured for 12 h. Then total RNAs of cells or lung tissues were extracted by using Trizol agent. Total RNAs were reverse-transcribed with oligo-dT primers