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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

(Takara). Quantitative real-time RT-PCR (qRT-PCR) was performed with the use of the Applied Lightcycler 2.0 detection system (Roche Applied Science, Germany) and the SYBR Green I reagent following the manufacturers' instructions. PCR program was as follows: 94 °C for 30 s, 40 cycles of 94 °C for 5 s, 56 °C for 30 s, 72 °C for 20 s. The primer pairs for Nox4 PCR (125 bp) were (forward) 5′-TGTTGGGCCTAGGATTGTGTT-3′ and (reverse) 5′-AGGGACCTTCTGTGATCCTCG-3′, and for the housekeeping gene GAPDH (359 bp) were (forward) 5′-TGAAGGTCGGTGTGAACGGATTTG-3′ and (reverse) 5′-GGCGGAGATGATGACCCTTTTG-3′, respectively.

2.13. Statistical analyses

Results were expressed as means ± S.E.M. Statistical analysis was performed by SPSS (Version 11.5, SPSS Inc., Chicago, USA). Comparisons between groups were performed using ANOVA with a Holms–Sidak *post hoc* test. Significant difference was accepted at $P<.05$.

3. Results

3.1. GSPE attenuated hypoxia-induced pulmonary artery remodeling and PH in rats

After exposure to hypoxia for 4 weeks, the average RVSP of hypoxia group was increased significantly compared with the normoxia group. However, the average RVSP of hypoxia treated with GSPE group was much lower than that of hypoxia alone group (Fig. 1A). In accordance with the RVSP, hypoxia-induced elevation of the ratio of RV/LV+S was inhibited by the application of GSPE (Fig. 1B). To investigate the effect of GSPE on hypoxia-induced pulmonary artery remodeling, WT% and WA% of pulmonary arteries which were stained by hematoxylin and eosin were evaluated. As shown in Fig. 2, hypoxia markedly elevated WT% and WA%, while WT% and WA% in hypoxia group treated with GSPE were much lower than in the hypoxia group.

3.2. GSPE reduced elevated expression of α-SMA in pulmonary arteries

To determine the cellular basis for the increased thickness and area of pulmonary arteries, the hyperplastic smooth muscularization in lung sections was evaluated using an antibody against α-SMA (Fig. 3). Integrated optical density (OD) value of α-SMA in hypoxia group was significantly higher than in normoxia group. Thus, increased thickness and area of pulmonary arteries are associated with enhanced proliferation of SMCs. With the treatment of GSPE, the integrated OD value of α-SMA was reduced significantly.

3.3. GSPE attenuated the hypoxia-induced oxidative stress in rats

Antioxidant enzyme SOD plays a critical role in the regulation of the oxidant levels in the vasculature, and its dysregulation has been implicated in the pathology of PH [23]. In addition, MDA is formed as an end product of lipid peroxidation and acts as a marker of endogenous lipid peroxidation. To determine GSPE's antioxidative effect, SOD and MDA were analyzed both in lung tissue and in serum (Fig. 4). The results showed that hypoxia decreased the levels of SOD, companied with a increased levels of MDA. GSPE treatment elevated the levels of SOD and reduced the levels of MDA both in lung tissue and in serum.

3.4. GSPE decreased the concentration of intracellular ROS

To investigate whether GSPE could reverse the hypoxia-induced up-regulation of ROS production in PASMCs, we used the general ROS indicator DCFDA to determine the level of intracellular ROS (Fig. 5). Flow cytometry results presented that chronic hypoxia could increase the level of intracellular ROS in PASMCs significantly. However, GSPE obviously inhibited ROS production, and this inhibitory effect on ROS production was enhanced with increasing GSPE concentrations.

3.5. GSPE reversed the hypoxia-induced up-regulation of Nox4 mRNA levels both in PASMCs and in lung tissue

Nox4 plays an important role in the pathogenesis of hypoxia-induced pulmonary arterial remodeling and PH [24]. During chronic hypoxia, Nox4 is prominently up-regulated in pulmonary arterial vessels and in SMCs [25]. To determine whether hypoxia modulates Nox4 gene expression and the possible effects of GSPE on Nox4, we assessed Nox4 mRNA levels both in PASMCs and in lung tissue (Fig. 6). Results confirmed that exposure to chronic hypoxia for 4 weeks increased the levels of Nox4 mRNA in rat lung, and treatment with GSPE prevented hypoxia-mediated Nox4 mRNA induction. Furthermore, hypoxia also increased the expression of Nox4 mRNA in PASMCs, and hypoxia-induced increases in Nox4 mRNA were reduced by treatment with GSPE.

3.6. GSPE inhibited hypoxia-induced PASMCs proliferation

Aiming to know whether GSPE could inhibit hypoxia-induced PASMCs proliferation, the Ki67-positive cells were detected primarily in the medial wall of pulmonary arteries (Fig. 7). The percentage of Ki67-positive cells in hypoxia group was notably higher than in normoxia group. However, increased cell proliferation in the hypoxia group was inhibited markedly by the treatment of GSPE. In addition, Fig. 8 showed that the proliferation of PASMCs was significantly elevated in hypoxia compared with the cells under normoxic conditions, while GSPE supplement exerted a significant

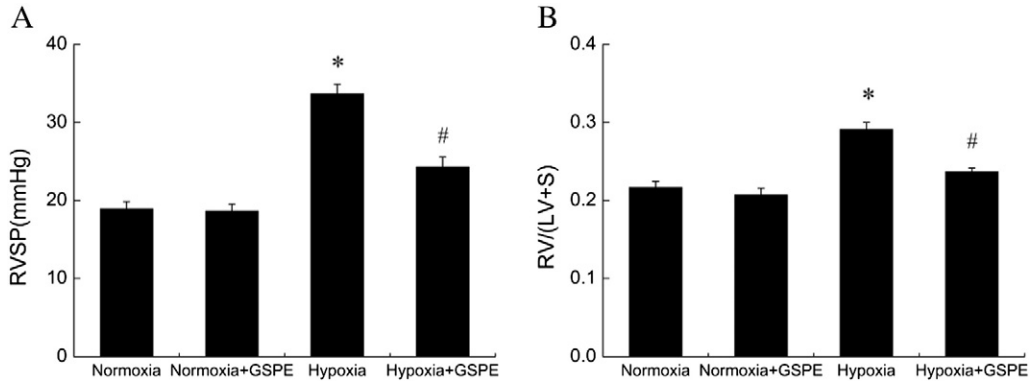

Fig. 1. Analysis of hemodynamic data and RV hypertrophy index. Right ventricular systolic pressure (RVSP) in rats exposed to normoxia or hypoxia (A). Weight ratio of the right ventricular wall (RV) and the left-ventricular wall (LV) and septum (S) [RV/(LV+S)] in rats exposed to normoxia or hypoxia (B). Values are means ± S.E.M., $^{*}P<.01$ compared with normoxia group. $^{\#}P<.01$ compared with hypoxia group ($n=7$).

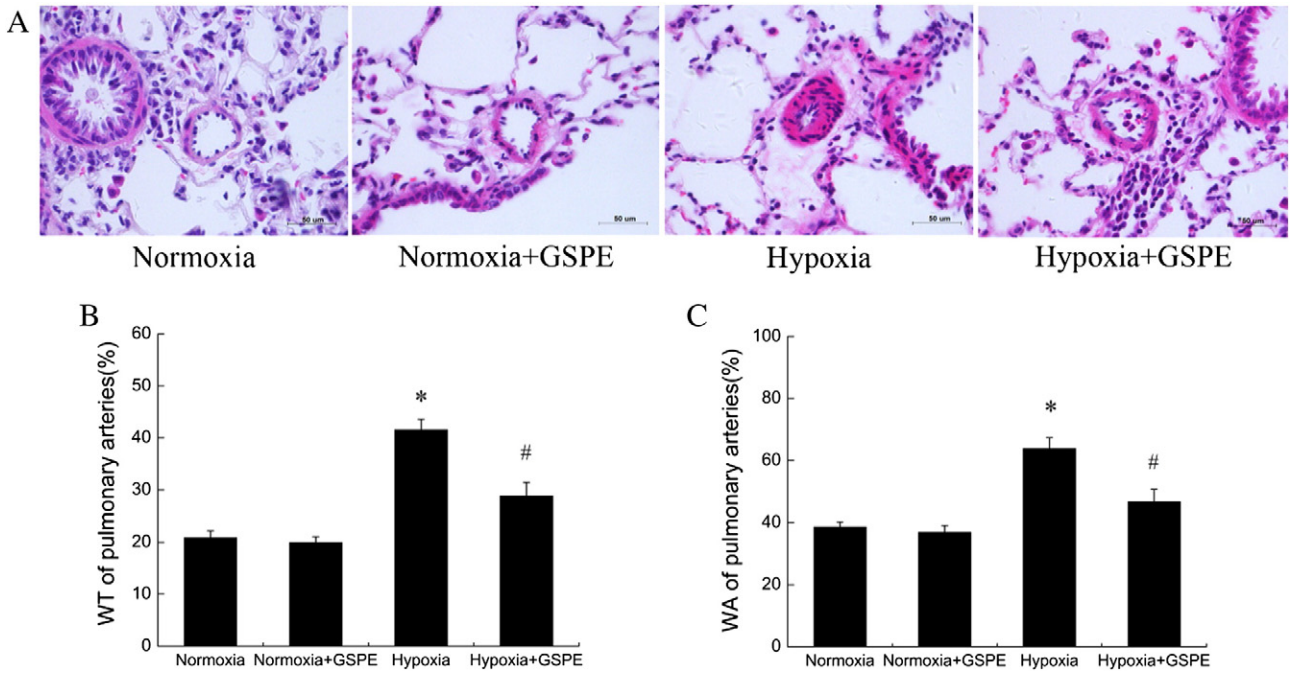

Fig. 2. Effects of GSPE on hypoxia-induced pulmonary vascular remodeling. Hematoxylin and eosin staining of pulmonary arteries (magnification ×400) (A). Percentage of medial (%) of pulmonary arteries (B). Percentage of medial wall area (WA%) of pulmonary arteries (C). Values are means ± S.E.M., $^{*}P<.01$ compared with normoxia group. $^{\#}P<.01$ compared with hypoxia group ($n=7$).

antiproliferative effect, and this inhibitory effect on PASMCs proliferation was enhanced with increasing GSPE concentrations.

3.7. GSPE reduced the protein expression of p-STAT3, cyclin D1 and cyclin D3 in PASMCs

To further investigate the cellular and molecular mechanisms underlying GSPE-induced PASMCs growth inhibition, we evaluated the protein levels of p-STAT3, cyclin D1 and cyclin D3 in PASMCs (Fig. 9). Western blotting results showed that hypoxia notably increased the p-STAT3, cyclin D1 and cyclin D3 protein levels in PASMCs. The expression of p-STAT3, cyclin D1 and cyclin D3 in the four dosages of GSPE treatment groups were all significantly lower compared with the hypoxia group.

4. Discussion

Through exposing rats to normobaric hypoxia for 4 weeks, we successfully established an animal model of hypoxia-induced PH. This rat model exhibited significant structural remodeling of pulmonary arteries and elevated pulmonary artery pressure. GSPE supplementation markedly repressed the elevation of RVSP and [RV/(LV+S)], as

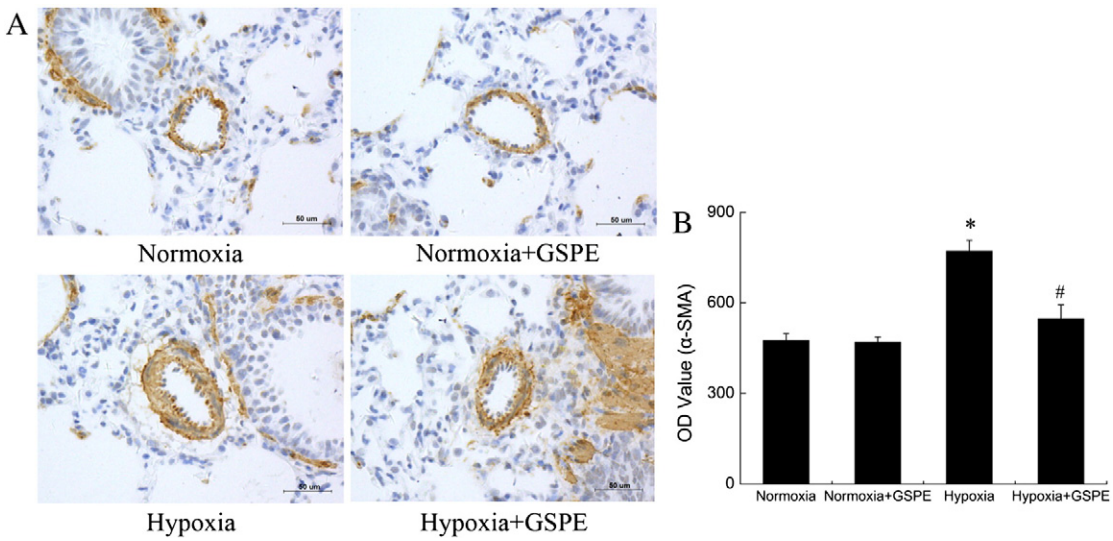

Fig. 3. Analysis of OD value of α-SMA in pulmonary arteries. Immunohistochemical staining of α-SMA of pulmonary arteries (magnification ×400) (A). Bar graph shows the quantitative analysis of OD value of α-SMA immunoreactivity in pulmonary arteries (B). Values are means ± S.E.M., $^{*}P<.01$ compared with normoxia group. $^{\#}P<.01$ compared with hypoxia group ($n=7$).

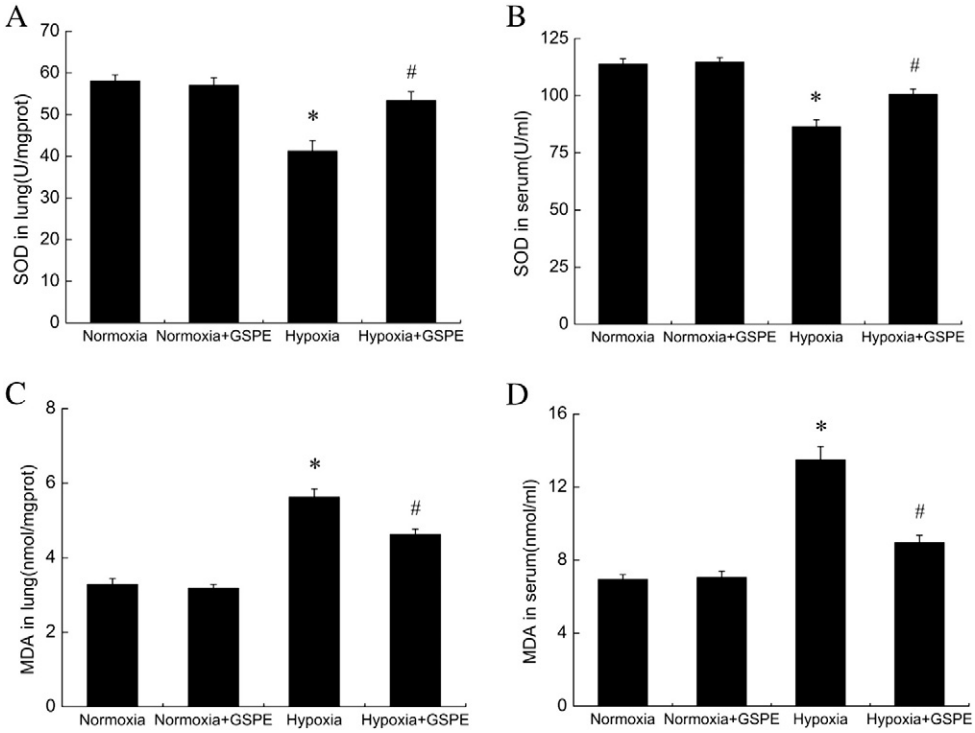

Fig. 4. Effects of GSPE on the levels of SOD and MDA. SOD in lung tissue (A). SOD in serum (B). MDA in lung tissue (C). MDA in serum (D). Values are means ± S.E.M., *P<.01 compared with normoxia group. #P<.01 compared with hypoxia group (n=7).

well as the increased WT% and WA% in hypoxic rats. Furthermore, the increased cellular proliferation in pulmonary arteries as evident by enhanced α-SMA, the marker for SMCs, immunoreactivity in pulmonary arteries was also inhibited by GSPE. These results indicated that GSPE could prevent pulmonary artery pressure elevation, right ventricular hypertrophy and vascular remodeling in a chronic hypoxia rat model.

Substantial evidence is accumulating to suggest that ROS-mediated oxidative damage plays an important role in the pathogenesis of PH. ROS modulates the effects of and/or release of several vasoactive factors, such as ET-1, TXA2 and prostacyclin, which can acutely influence vessel tone and result in vascular remodeling in PH [23,24]. Moreover, ROS specifically has been implicated in both a rat and mouse model of chronic hypoxia-induced PASMCs remodeling and PH, and inhibition of ROS production has been shown to attenuate HPH [26]. To explore whether GSPE's protection was related to antioxidative effect, we determined the levels of antioxidant enzyme SOD and lipid peroxidation marker MDA in lung tissue and in serum. SOD protects the cells against the potential damage from superoxide radicals *via* catalyzing the conversion of superoxide radical to

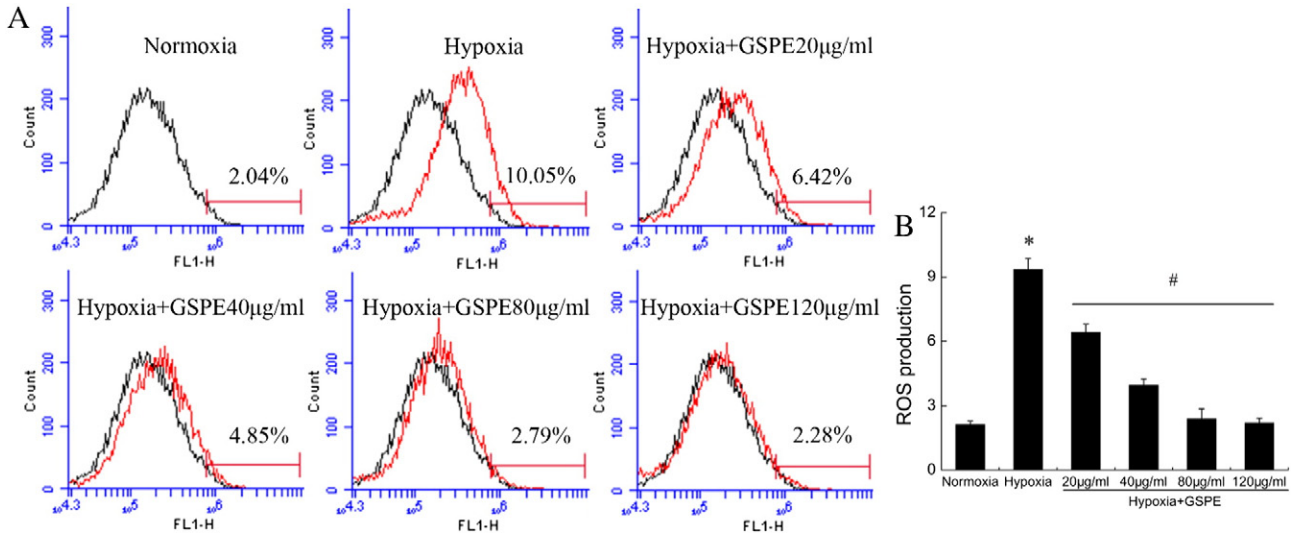

Fig. 5. Effects of GSPE on hypoxia-induced ROS Production in PASMCs. Production of ROS in PASMCs was measured in different concentrations of GSPE under normoxic or hypoxic conditions (A). Bar graph shows the statistic results of ROS production (B). Values are means ± S.E.M., *P<.01 compared with normoxia group. #P<.01 compared with hypoxia group (n=3).

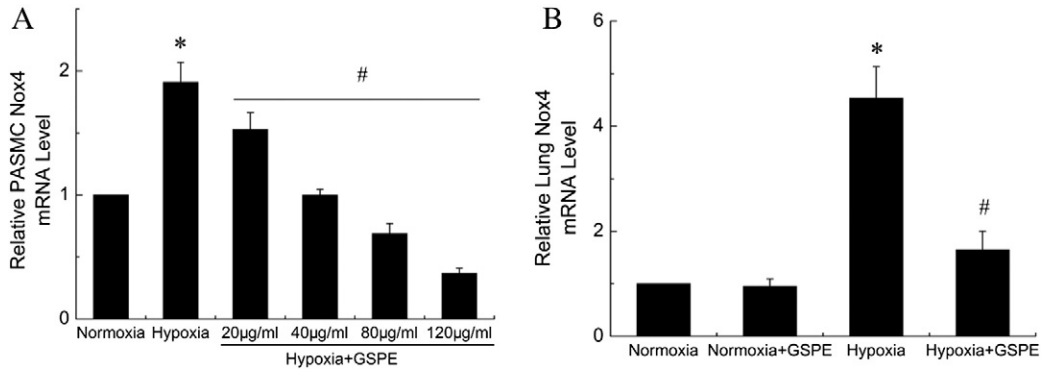

Fig. 6. Effects of GSPE on Nox4 mRNA expression in rat lungs and PASMCs. Relative expression of Nox4 mRNA levels in PASMCs in different concentrations of GSPE under normoxic or hypoxic conditions (A). Relative expression of Nox4 mRNA levels in rat lungs (B). Values are means ± S.E.M., $^{*}P<.01$ compared with normoxia group. $^{\#}P<.05$ compared with hypoxia group ($n=3$).

hydrogen peroxide [27]. There are three known forms of SOD: CuZn-SOD (SOD1), Mn-SOD (SOD2) and EC-SOD (SOD3) that all serve to catalyze the rapid conversion of superoxide radical to hydrogen peroxide [28]. In PH of different etiologies, the changes in expression and/or activity of these isoforms contribute to oxidative stress and vascular remodeling [23]. In addition, recent reports have implicated increased lipid peroxidation as a mediator in the pathogenesis and the development of PH, and antilipid peroxidation treatment therapy has been effective in the treatment of pulmonary arterial pressure and pulmonary resistance in PH [29,30]. In this study, results showed that GSPE elevated the decreased levels of SOD and reduced the increased levels of MDA caused by hypoxia both in lung tissue and in serum. *In vitro*, we used primary PASMCs to evaluate the antioxidative effect of GSPE. Considerable evidence has supported that ROS is paradoxically increased in PASMCs under hypoxia [13,25]. Accordingly, our data showed that chronic hypoxia increased the level of intracellular ROS in PASMCs significantly. Moreover, GSPE markedly inhibited ROS production. These results suggested that GSPE could attenuate the hypoxia-induced oxidative stress in HPH.

Although several enzymes are now recognized to produce ROS, perhaps the most important of these is NADPH oxidase which plays critical roles in both vasoconstrictive and remodeling aspects of PH [23]. Seven Nox enzymatic subtypes have been identified in a wide range of cell types: Nox1–5 and Duox1–2, but only Nox1, Nox2, Nox4 and Nox5 are found in the pulmonary vasculature [11,24]. Among them, Nox4 is highly expressed and has been reported to be the

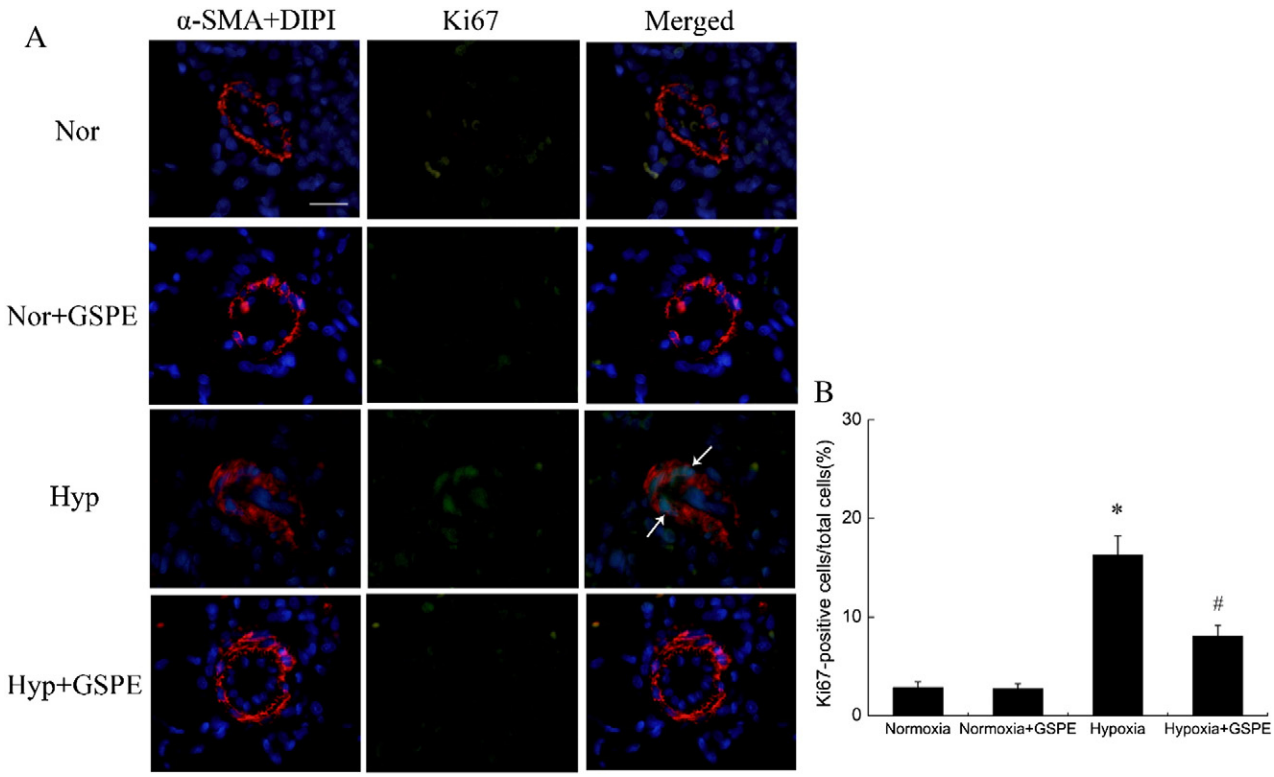

Fig. 7. Analysis of the percentage of Ki67-positive cells in the medial wall of pulmonary arteries. Immunofluorescence staining of Ki67 of pulmonary arteries (A). Scale bar: 20 μm. Bar graph shows that the number of Ki67-positive cells relative to the total SMCs in the medial wall of pulmonary arteries (%) (B). "Nor" means normoxia; "Hyp" means hypoxia. Values are means ± S.E.M., $^{*}P<.01$ compared with normoxia group. $^{\#}P<.01$ compared with hypoxia group ($n=7$).

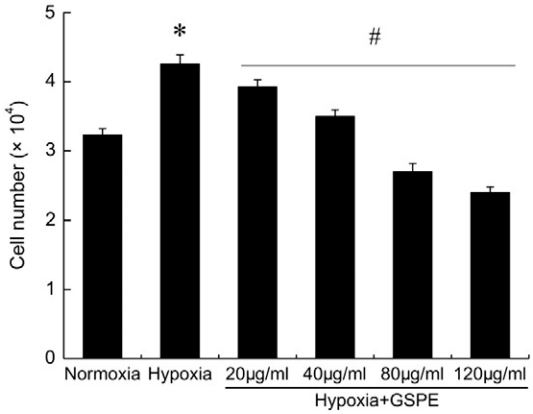

Fig. 8. Effects of GSPE on PASMCs proliferation under hypoxia exposure. Proliferation of PASMCs was measured in different concentrations of GSPE under normoxic or hypoxic conditions. Values are means ± S.E.M., *P<.01 compared with normoxia group. #P<.05 compared with hypoxia group (n=3).

predominant homolog in PASMCs [31,32]. Hypoxia up-regulates Nox4 mRNA and protein levels in SMCs, while siRNA-mediated silencing of Nox4 reduces ROS and cellular proliferation, suggesting that Nox4 mediates pulmonary vascular remodeling [25]. Moreover, it has been confirmed that hypoxia is a stimulus for increasing expression of Nox4 in PASMCs leading to increased ROS generation and resultant cell proliferation [13]. In the present investigation, we observed that Nox4 was up-regulated by hypoxia at the mRNA levels in PASMCs. Furthermore, Nox4 mRNA levels were also enhanced in lung tissue from rat exposed to hypoxia. However, GSPE significantly inhibited the increased expression of Nox4 mRNA levels caused by hypoxia.

For the ROS promoting PASMCs proliferation in response to hypoxia and the possible effect of GSPE on PASMCs proliferation, we evaluated Ki67, STAT3 and its target gene products, cyclin D1 and cyclin D3 in PASMCs. The signal transducer and activator of transcription 3 (STAT3) has been extensively described as a central signaling molecule that is implicated in the regulation of several genes involved in cell proliferation, differentiation and survival [33,34]. Several studies have shown that ROS as second messenger molecules may activate STAT3, and activated STAT3-dependent pathway was involved in the process of hypoxia-induced PASMCs proliferation and vascular remodeling in PH [33,35,36]. In addition, cyclin D1 and cyclin D3 were also found to be up-regulated in PH, which play an important role in regulating the transition from G1 to S phase during the cell cycle [35]. In this study, western blotting and immunofluorescence staining results showed that the increased expression of phospho-STAT3, cyclin D1, cyclin D3 and Ki67 in PASMCs caused by hypoxia was significantly down-regulated by GSPE. These results suggested that ROS/STAT3/cyclins pathway may participate in the PASMCs proliferation caused by hypoxia. The exact mechanism of inhibition of PASMCs proliferation by GSPE and the association between ROS and STAT3, cyclin D1 and cyclin D3 signaling need to be further investigated.

Currently, the effect of GSPE on the prevention and treatment of cardiovascular diseases has been confirmed. GSPE possesses a broad spectrum of biological effects including antioxidant, antiinflammation, antiatherosclerosis and others.[37,38]. Moreover, GSPE is a popular herbal supplement with patients suffering from cardiovascular disease. Although GSPE has multiple beneficial effects on the cardiovascular system, to our knowledge, this is the first report that GSPE directly attenuates HPH development *in vivo*.

In conclusion, GSPE supplementation prevents the changes in hemodynamics and pulmonary vascular remodeling that occur in rats exposed to hypoxia. Moreover, our data also show that GSPE could attenuate the oxidative stress and the proliferation of PASMCs caused by hypoxia. These findings provide clues that GSPE might be used to prevent HPH and open new perspectives in the field of prevention of HPH.

Acknowledgments

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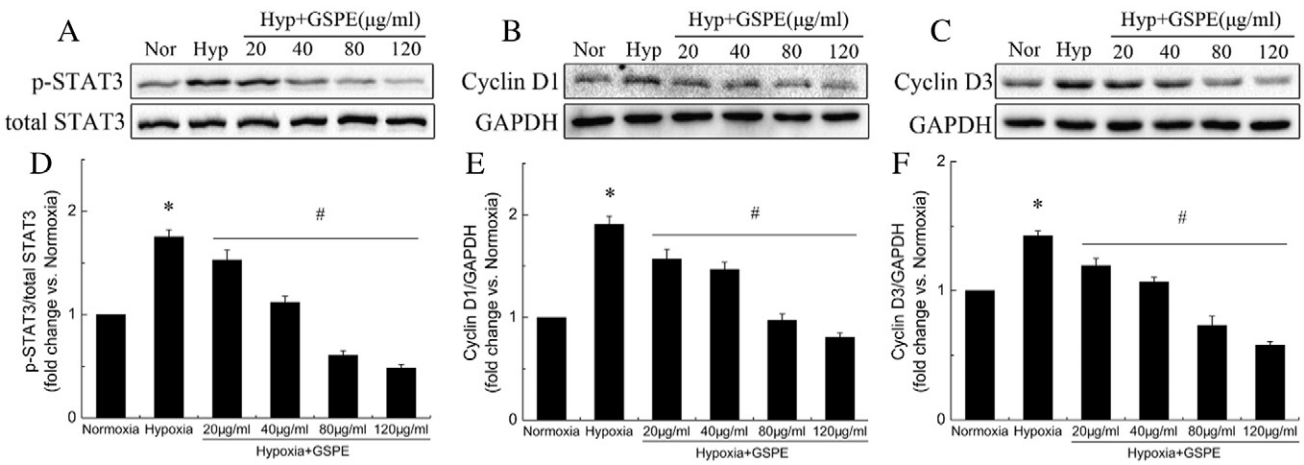

Fig. 9. Effects of GSPE on p-STAT3, cyclin D1 and cyclin D3 expression in PASMCs. Representative western blotting analysis of p-STAT3, cyclin D1 and cyclin D3 protein levels in PASMCs in different concentrations of GSPE under normoxic or hypoxic conditions (A, B, C). Bar graph shows the statistic results of p-STAT3, cyclin D1 and cyclin D3 protein levels, respectively (D, E, F). "Nor" means normoxia; "Hyp" means hypoxia. Values are means ± S.E.M., *P<.01 compared with normoxia group. #P<.05 compared with hypoxia group (n=3).

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