Astragalus polysaccharide ameliorates H$_2$O$_2$-induced human umbilical vein endothelial cell injury

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Abstract. Endothelial dysfunction caused by reactive oxygen species (ROS) has been implicated in numerous cardiovascular diseases. Astragalus polysaccharide (APS), an important bioactive component extracted from the Chinese herb Astragalus membranaceus, has been widely used for the treatment of cardiovascular disease. The present study aimed to investigate the effects of APS on hydrogen peroxide (H$_2$O$_2$)-induced human umbilical vein endothelial cell (HUVEC) injury. Following treatment with 400 µM H$_2$O$_2$ for 24 h, cell viability was decreased and apoptosis was increased. However, pretreatment with APS for 1 h significantly attenuated H$_2$O$_2$-induced injury in HUVECs. In addition, APS decreased intracellular ROS levels, increased the protein expression of endothelial nitric oxide synthase and copper-zinc superoxide dismutase, elevated intracellular cyclic guanosine monophosphate (an activity marker for nitric oxide) levels and restored the mitochondrial membrane potential, compared with cells treated with H$_2$O$_2$ only. In conclusion, the results of the present study suggested that APS may protect HUVECs from injury induced by H$_2$O$_2$ via increasing the cell antioxidant capacity and nitric oxide (NO) bioavailability, which may contribute to the improvement of the imbalance between ROS and NO levels.

Introduction

Vascular endothelial cells are flat cells lining the inner wall of blood vessels, which serve as a barrier between circulating blood and smooth muscle cells. In addition, the vascular endothelium is an important endocrine organ, which is involved in maintaining vascular wall tension, blood flow, vessel wall inflammation antagonism and angiogenesis through the secretion of numerous vasoactive substances. Due to their barrier function, endothelial cells are more vulnerable to injury by various physical and chemical risk factors. Injury to endothelial cells is a critical event in angiogenesis, atherosclerosis, thrombosis, hypertension and heart failure (1-5). Endothelial dysfunction, particularly endocrine dysfunction, causes the secretion of a variety of active substances, which may lead to dysfunction of the cardiovascular system.

In endothelial cells, oxidative stress is regarded as a critical pathogenic factor for endothelial cell injury, and the accumulation of ROS may result in endothelial cell apoptosis (6-8). Hydrogen peroxide (H$_2$O$_2$) may penetrate the plasma membrane and cause endothelial cell injury. In addition, it has been reported that reactive oxygen species (ROS) are involved in the apoptosis of endothelial cells (9,10) and the primary source of endogenous ROS is H$_2$O$_2$ (11), which has been extensively used to induce oxidative stress in in vitro models (12,13).

Radix astragali is the root of the perennial herb Astragalus membranaceus (14). As a well-established traditional Chinese medicine, it has been used for the treatment of cardiovascular disease and is believed to possess immune stimulatory, antiviral and antioxidative effects. Astragalus polysaccharide (APS) is an important bioactive ingredient obtained from Astragalus membranaceus that has a range of pharmacological effects, including increasing levels of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) in plasma and tissues, promotion of immune responses, anti-inflammation (15), protection of vessels, antioxidant effects, anti-insulin resistance and anti-tumor effects (16-19). Our previous studies reported that APS may inhibit isoprenaline-induced cardiac hypertrophy (20,21). It has been reported that Astragalus membranaceus and its primary components, including APS, may ameliorate...
endothelial dysfunction induced by homocysteine in which antioxidation is involved. However, the effects of APS on endothelial injury induced by ROS and the underlying mechanism remains to be fully elucidated.

Materials and methods

Materials. APS was purchased from Nanjing Jingzhu Pharmaceutical Technology Co., Ltd. (Nanjing, China). H₂O₂ was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Dihydroethidium (DHE) was obtained from Shanghai Haoran Bio-Technology Co., Ltd. (Shanghai, China). MTT and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). DAPI, Rhodamine 123 and the Cell Cycle and Apoptosis analysis kit were purchased from Beyotime Institute of Biotechnology (Haimen, China). The cGMP assay kit was supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rabbit anti-Copper-zinc superoxide dismutase (Cu/Zn-SOD; bs-10216R) and anti-endothelial nitric oxide synthase (eNOS; bs-20609R) antibodies were purchased from Beijing Boosen Biological Technology Co., Ltd (Beijing, China), and anti-β-actin (HRP-600008) was from Wuhan Biotechnology (Wuhan, China). All other chemicals and reagents used were of analytical grade.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were obtained from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China) and were maintained in high-glucose Dulbecco’s Modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 2 mmol/l L-glutamine, 5 µg/ml streptomycin. Cells were incubated in a humidified incubator dark. Cells were analyzed under an inverted fluorescence microscope.

Measurement of intracellular cGMP levels. HUVECs were cultured with 0.1, 1 or 10 µg/ml APS for 1 h, followed by exposure to 400 µM H₂O₂ for 24 h. The cells were subsequently harvested with a Falcon scraper, centrifuged at 4°C for 5 min at 800 x g and sonicated. The homogenate was prepared in PBS and centrifuged at 1,000 x g for 5 min at 4°C. The cGMP levels in the supernatants were determined with a kit according to the manufacturer’s protocol. cGMP levels were expressed as nmol/g protein. Protein concentration was calculated with a Bicinchoninic Acid (BCA) Protein assay kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China). cGMP is a second messenger of NO, and its level reflects the level of nitric oxide (NO) (27).

Measurement of intracellular ROS production. The production of ROS was measured using DHE. DHE, an oxidative fluorescent dye, may be oxidized to ethidium bromide and intercalated into DNA in the presence of superoxide anions. HUVECs were seeded on a 6-well plate (1x10⁵ cells/well).
Results

Effects of APS on the viability of HUVECs. Initially, the cytotoxicity of APS on HUVECs was measured using the MTT assay. As presented in Fig. 1A, incubation of HUVECs with 0.1-100 μg/ml APS for 24 h did not significantly affect the cell viability (P>0.05). However, treatment of HUVECs with 100 to 500 μM H₂O₂ for 24 h resulted in a concentration-dependent decrease in cell viability, when compared with the control group (P<0.05; Fig. 1B). Pretreatment of HUVECs with 0.1, 1 or 10 μg/ml APS for 1 h prior to exposure to H₂O₂ significantly increased viability in a dose-dependent manner (Fig. 1C). These results indicated that APS may protect HUVECs from oxidative stress-induced injury. Based on these results, further studies employed a H₂O₂ concentration of 400 μM, and APS concentrations of 0.1, 1.0 and 10 μg/ml.

Effect of APS on H₂O₂-induced apoptosis in HUVECs. To further evaluate the protective effect of APS against H₂O₂-induced damage in HUVECs, apoptosis rates were measured by flow cytometry. As presented in Fig. 2, the percentage of apoptotic cells in the control group was 5.44±0.57%. Following exposure to 400 μM H₂O₂ for 24 h, the percentage of apoptosis increased to 43.14±4.25%. However, pretreatment of the cells with 0.1, 1 and 10 μg/ml APS for 1 h prior to H₂O₂ exposure reduced the percentage of apoptotic cells to 38.58±4.13, 27.60±2.25 and 24.10±3.26%, respectively. In addition, the morphology of HUVEC nuclei was evaluated by DAPI staining. Compared with the control group (Fig. 3A), nuclei of the H₂O₂ group (Fig. 3B) were shrunken, irregular and fragmented, typical features of apoptosis. However, APS (1 μg/ml) pretreatment (Fig. 3C) reduced these alterations.

Effect of APS on the mitochondrial membrane potential of HUVECs. The disruption of mitochondria has been reported to be involved in programmed cell death. The maintenance of the mitochondrial membrane potential is essential for

Following treatment, cells were washed with PBS and incubated with 2 mM DHE for 30 min at 37°C. Cells were washed with PBS and analyzed under an inverted fluorescence microscope. The fluorescence intensity was calculated using LAS Software v4.3.

Western blot analysis. Following treatment, cells were harvested and lysed with ice-cold radio immunoprecipitation assay buffer (Wuhan Boster Biotechnology Co., Ltd., Wuhan, China). Protein concentration was determined using a BCA Protein assay kit. Protein extracts (30 μg) were electrophoretically separated by 12 or 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk powder and incubated overnight at 4°C with the following primary antibodies: Anti-eNOS (1:1,500), anti-Cu/Zn-SOD (1:500) and β-actin (1:500). Following washing with TBS containing Tween-20 (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20), membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG and anti-mouse IgG; 1706515 and 1706516; 1:1,500; Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 2 h at room temperature. Membranes were washed with TBS, and immunocomplexes were visualized using the Amersham ECL start Western Blotting Detection Reagent enhanced chemiluminescence system (GE Healthcare Life Sciences). β-actin served as an internal control for the experiments. The results were quantified by the Quantity One software v4.62 (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data are expressed as the mean ± standard deviation. Each experiment was performed at least three times. Statistical analysis was performed using Student's t-test or one-way analysis of variance with SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). The post hoc least significant difference test was used to evaluate differences between two groups. P<0.05 was considered to indicate a statistically significant difference.
mitochondrial integrity and bioenergetic function. To determine whether the anti-apoptotic effect of APS is associated with the inhibition of mitochondrial disruption, mitochondrial membrane potential variation in HUVECs was assessed by Rhodamine 123 staining. Rhodamine 123 is sequestered by active mitochondria of normal HUVECs and revealed yellow-green flavovirens fluorescence. Compared with control cells (Fig. 4A), H₂O₂-treated cells presented a weak green

Figure 2. Apoptosis of human umbilical vein endothelial cells, as determined by flow cytometry. (A) Control, (B) H₂O₂ only, (C) 0.1 µg/ml APS + H₂O₂, (D) 1 µg/ml APS + H₂O₂, and (E) 10 µg/ml APS + H₂O₂ groups. (F) The effect of APS on the percentage of apoptotic cells induced by 400 µM H₂O₂. Data are expressed as the mean ± standard deviation. #P<0.01 vs. control; ##P<0.01 vs. H₂O₂ only. APS, Astragalus polysaccharide.
fluorescence intensity, which reflected the loss of mitochondrial membrane potential (Fig. 4B); this decrease in fluorescence was partially reversed by APS (1 µg/ml) pretreatment (Fig. 4C). Quantification of fluorescence intensity revealed that these alterations were significant (Fig. 4D). This may be a possible underlying mechanism by which APS protects against injury.

Effect of APS on cGMP levels in HUVECs. To further investigate the protective effects of APS, the influence of H_{2}O_{2} on cGMP accumulation was measured. As presented in Fig. 5, basal production of cGMP decreased from 9.8±0.8 nmol/g protein in control cells to 4.2±0.4 nmol/g protein in H_{2}O_{2}-treated cells. However, pretreatment of cells with 0.1-10 µg/ml APS for 1 h prior to H_{2}O_{2} exposure increased the levels of cGMP compared with H_{2}O_{2} treatment alone, in a dose-dependent manner.

Effect of APS on ROS formation in HUVECs. The secondary generation of ROS is another potential mechanism underlying cell damage induced by H_{2}O_{2}. Therefore, intracellular ROS levels were measured by DHE staining. Compared with the control group (Fig. 6A), DHE fluorescence in HUVECs...
exposed to H$_2$O$_2$ was increased (Fig. 6B). This increase was attenuated by pretreatment with APS (Fig. 6C). Quantification of fluorescence revealed significantly increased ROS production in H$_2$O$_2$-treated cells (P<0.01), and a significant and dose-dependent effect of APS (P<0.01; Fig. 6D).

**Effect of APS on the protein expression levels of eNOS and Cu/Zn-SOD in H$_2$O$_2$-treated cells.** The protein expression levels of eNOS and Cu/Zn-SOD were examined to investigate the potential underlying molecular mechanisms contributing to APS cytoprotection. Protein expression levels of Cu/Zn-SOD (Fig. 7A) and eNOS (Fig. 7B) in HUVECs exposed to H$_2$O$_2$ were significantly decreased (P<0.01). However, pretreatment with 10, 1 or 0.1 µg/ml APS significantly attenuated the H$_2$O$_2$-mediated decrease of eNOS and Cu/Zn-SOD expression (P<0.01), which may therefore contribute to the APS-mediated protective effects of HUVECs.

**Discussion**

Vascular endothelial cells form a crucial functional barrier between tissues and the circulation, which acts as a receptor
that senses physical or chemical stimuli occurring inside the vessel. In addition, this barrier may secrete a variety of substances that contribute to the response of the blood vessel to different physiologic and pathologic stimuli, including regulation of coagulation and participation in the immune response. Abnormalities in endothelial cell structure and function potentially lead to numerous cardiovascular diseases. Therefore, endothelial function has been identified as a biomarker/mediator of cardiovascular risk factors (28-30) that may act as an independent predictor of cardiovascular disease (3,31). APS, isolated from Astragalus membranaceus, has a variety of pharmacological effects, including anti-inflammatory, antiviral and antioxidant effects, and it increases levels of cGMP and cAMP in plasma and tissues (32). It has been reported that APS has cytoprotective effects on the erythroid lineage K562 cells (33). In addition, APS may improve the response of C2C12 skeletal muscle myotubes and myoblasts to peroxide-induced injury in vitro by inhibiting apoptosis (34). However, the effect and underlying mechanisms of APS on H$_2$O$_2$-induced injury in HUVECs remain to be elucidated.

In the present study, the effects of APS on H$_2$O$_2$-induced injury in HUVECs in vitro and the possible underlying mechanisms were investigated. The results indicated that APS protected HUVECs from H$_2$O$_2$-induced apoptosis. Furthermore, APS markedly decreased intracellular ROS levels, increased protein expression levels of eNOS and Cu/Zn-SOD, partially restored the mitochondrial membrane potential and increased levels of cGMP, compared with cells treated with H$_2$O$_2$ alone. The underlying mechanisms of APS against HUVEC apoptosis may involve an increase in antioxidant defense systems and an increase in the production and bioavailability of NO.

NO, an endothelium-dependent vasodilator, is an important mediator in the regulation of endothelial cell function. In addition, it has numerous biochemical activities, including directly scavenging superoxide, maintaining endothelial integrity, attenuating leukocyte adhesion and inhibiting platelet aggregation (5). Endothelial NO produced by eNOS may inhibit apoptosis, and is regarded as a survival factor for endothelial cells (35). It has been reported that the reduced formation of NO or impairment of NO effects may be associated with aortic sclerosis (36). Additional studies have indicated that reduced NO bioavailability enhances atherogenesis in animal models (8), and contributes to endothelial dysfunction (37). In the current study, the intracellular cGMP levels were detected to evaluate the bioavailability of NO, as it is the second messenger of NO. The results suggested that APS pretreatment may increase the levels of cGMP and the protein expression levels of eNOS, compared with cells treated with H$_2$O$_2$ alone. This indicated that APS may protect HUVECs from injury induced by H$_2$O$_2$ via increasing the production and bioavailability of NO.

ROS, the free radicals present in all vascular cells, have been demonstrated to serve an important role in endothelial injury (38). Numerous studies have suggested that ROS are involved in vascular remodeling and endothelial dysfunction (39). Endothelial cells express a variety of enzymes from which ROS may be generated. Therefore, endothelial cells are regarded as an important source of vascular ROS production (40). In addition, mitochondria are important physiological sources of ROS. Furthermore, it has been reported that H$_2$O$_2$ may cause endothelial cell injury by inducing mitochondrial dysfunction, including loss of mitochondrial membrane potential (41).

Reduced bioavailability of NO contributes to endothelial dysfunction. The overproduction of ROS may inactivate NO by reacting with it to decrease NO synthesis and reduce NO bioavailability. Previous studies have suggested that the imbalance between ROS and NO levels, rather than the levels of each, may be a primary cause of endothelial dysfunction in numerous cardiovascular diseases. The results of the present study suggested that APS may protect endothelial cells against injury induced by H$_2$O$_2$ via increasing the protein expression levels of Cu/Zn-SOD. This may lead to superoxide scavenging, restoring mitochondrial membrane potential and decreasing the intracellular ROS levels detected by DHE staining.

In conclusion, the present study demonstrated that APS had a protective effect against H$_2$O$_2$-induced injury and apoptosis in HUVECs. The underlying mechanisms of the protective effects of APS may involve restoration of the balance between ROS and NO levels via increasing the cell antioxidant capacity and NO bioavailability.

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