RETRACTED ARTICLE: Astragalus polysaccharide alleviates H$_2$O$_2$-triggered oxidative injury in human umbilical vein endothelial cells via promoting KLF2

Dongtao Li, Yan Liu, Rong Xu, Xin Jia, Xing Li, Cong Huo and Xiaoming Wang

Department of Geriatrics, Xijing Hospital, The Fourth Military Medical University, Xi’an, China

ABSTRACT
The damage of vascular endothelial cells has become an indispensable factor in the occurrence and advancement of cardiovascular diseases. In the current study, we investigated the effect of Astragalus Polysaccharin (APS) on H$_2$O$_2$-evoked oxidative injury in HUVECs. HUVECs cells were treated by H$_2$O$_2$ to induce oxidative damage. Cells viability, apoptosis and reactive oxygen species (ROS) level were detected through CCK8 assay and flow cytometry. The cell growth-related proteins and home oxygenase-1(HO-1) and KLF2 expression were evaluated via Western blot assay. The functions of KLF2 in APS and H$_2$O$_2$ co-disposed HUVECs were explored after si-KLF2 transfection. MEK/ERK pathway was finally measured through Western blot. We found that H$_2$O$_2$ stimulation-evoked HUVECs oxidative damage meanwhile impeded HO-1 expression. APS treatment effectively suppressed H$_2$O$_2$-induced oxidative injury in HUVECs. KLF2 and Nrf2 expression were elevated by APS and KLF2 repression abolished the protective action of APS in H$_2$O$_2$-triggered cell injury. MEK/ERK pathway was activated by APS treatment. Furthermore, the MEK/ERK pathway inhibitor weakened the promoting effect of APS on the expression of KLF2. In conclusion, our study reveals that APS alleviates H$_2$O$_2$-triggered oxidative injury in HUVECs via elevating the expression of KLF2 via the MEK/ERK pathway.

Introduction
Vascular endothelial cells (VECs) are monolayer epithelial cells in the medial part of all blood vessels, which play an important role in regulating vascular function [1]. Vasculature distributes in almost all organs and tissues and emerges a fundamental role in the function of organs and tissues [2]. Thus, vascular endothelial cell dysfunction has become a key factor in the occurrence and development of many diseases, including hypertension, coronary heart disease and other cardiovascular diseases [3,4]. Thrombosis caused by vascular endothelial injury is the pathological basis of many cardiovascular diseases. Oxidative stress, oxidized low-density lipoprotein, renin-angiotensin system and other factors can induce vascular endothelial cell injury [5]. Oxidative stress damages vascular endothelial cells by promoting the growth of inflammatory cells, inducing endothelial cell apoptosis, elevating the overexpression of inflammatory factors and degrading extracellular matrix [6]. The damage of vascular endothelial cells induced by oxidative stress is chiefly adjusted via reactive oxygen species (ROS). ROS can regulate the concentration of calcium ion in cytoplasm and the production of nitric oxide (NO), weaken vasodilation and lead to vascular endothelial damage [7]. Therefore, how to alleviate oxidative stress injury of endothelial cells is the key means to prevent and treat various vascular dysfunction-related diseases.

Recent studies have found that Astragalus mongholicus contains Astragalus polysaccharin (APS, molecular formula: C$_{10}$H$_7$ClN$_2$O$_2$S; molecular weight: 254.69), saponins, flavonoids, amino acids and some other active ingredients [8]. These active ingredients have the effect of promoting antibody production and immune response [9]. Among them, studies have corroborated that APS has an impact on antioxidant, anti-inflammatory and increase endothelial cell activity [10,11]. Herein, we attempted to probe the modulatory functions of APS in oxidative stress injury in HUVECs.

Krüppel-like transcription factors (KLF), as a kind of transcription regulatory proteins, participates in the transcription regulation process of various gene expressions, including inflammation, proliferation, growth, apoptosis, cell differentiation [12]. There are 17 members of the KLF family, named as KLF1-KLF17, respectively [12]. Among which, KLF2 was first cloned by Anderson in 1995 and it was found to be highly expressed in lung tissue and named as lung Krüppel-like factor [13]. Previous study has found that KLF2 is involved in regulating embryonic development, erythrocyte differentiation, inhibiting adipocyte differentiation and maintaining the resting state of T cells [14]. In recent years, emerging evidence has testified that KLF2 reveals an extraordinary role in adjusting endothelial cell biological activity and has become a hot topic in cardiovascular disease research [15]. In our...
present study, we also investigated whether the protective activity of APS in hydrogen peroxide (H$_2$O$_2$)-triggered oxidative injury in HUVECs is mediated by KLF2.

In our study, we discovered that APS effectively alleviated H$_2$O$_2$-triggered oxidative injury in HUVECs through rescuing cell viability, suppressing cell apoptosis and ROS and elevating HO-1 expression. Further study revealed that APS exerted the protective effect through elevating KLF2 expression via adjusting the MEK/ERK signal pathway. These findings provided novel therapeutic methods and targets for vascular dysfunction-related diseases.

**Materials and methods**

**Cell culture and treatment**

HUVECs were achieved from American Type Culture Collection (ATCC, Manassas, VA, USA) and were fostered in frequently-utilized RPMI 1640 medium with 10% FBS (Gibco, Waltham, MA, USA) in a moisten incubator containing 5% CO$_2$ at 37°C. Cells were treated by H$_2$O$_2$ at 5, 10, 50, 100, 200 and 300 μM concentrations for 24 h. APS (Boster Biology Co., Wuhan, China, purity >98.5%) was dissolved in aseptic phosphate buffer saline (PBS) for in vitro experiments. APS at the concentrations of 50, 100, 200 and 300 μM was adopted to deal with HUVECs for 24 h. The ERK inhibitor (SCH772984, 50 μM) was also utilized to dispose HUVECs for 24 h in the follow-up related experiments.

**Cell counting kit-8 (CCK-8) assay**

In brief, HUVECs were cultivated in 96-well plate and were fostered in a 5% CO$_2$ incubator at the constant 37°C temperature overnight. Afterward, CCK-8 (10 μl, Dojindo, Gaithersburg, MD, USA) solution was exploited for co-fostered with HUVECs for 1 h at 37°C. The absorbance assessment at 450 nm was evaluated through executing BioTek Synergy HT reader apparatus (BioTek, Winooski, VT, USA).

**Apoptosis assay**

HUVECs were rinsed in PBS and were then re-suspended in 400 μl binding buffer. Subsequently, HUVECs were stained with 5 μl PI/FITC-Annexin V (Biosea, Beijing, China) comprising 50 μg/ml RNase A (Sigma, St Louis, MO, USA) and then co-cultivated for 1 h in the blackness in consonance with the instruction. Flow cytometry analysis was executed through carrying out a flow cytometer (Beckman Coulter, Fullerton, CA, USA). The data in this experiment were analyzed through employing FlowJo software (TreeStar, Ashland, OR, USA).

**Western blot assay**

The proteins in HUVECs were extracted adopting RIPA lysis buffer (Beyotime, Shanghai, China) and the extracted protein concentrations were assessed through taking advantage of BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Then, the equal above protein samples were separated by 10% SDS-PAGE and electro-blotted onto PVDF membranes (Millipore, Billerica, MA USA). After blocking, the membranes were incubated with primary antibodies, which were Heme oxygenase-1 (HO-1, ab137749), p53 (ab131442), p21 (ab109199), Bax (ab53154), Cleaved-Caspase-9 (ab2324), Cleaved-Caspase-3 (ab49822), KLF2 (ab139699), p-MEK (ab254095), t-MEK (ab32091), p-ERK (ab201015), t-ERK (ab184699), β-actin (ab8227) and secondary antibody (ab205718, Abcam, Cambridge, UK). At last, the signals were apprehended and the intensity of the bands was assessed through employing ChemiDoc Imaging system (Bio-Red, Hercules, CA, USA).

**ROS assay**

ROS level was evaluated through employing flow cytometry along with 2,7-dichlorofluorescein diacetate (DCFH-DA) (Nanjing Jinheng, Nanjing, China). HUVECs were fostered into a 6-well plate after stimulation and transfection, which were then rinsed twice with PBS and were co-fostered with serum-free culture medium encompassing 10 μM DCFH-DA for 20 min at 37°C under the darkness. Subsequently, HUVECs were rinsed with PBS again and a trypsin digestion method was carried out for gathering the samples. The all the above gathered samples were centrifuged and the supernatants were removed. HUVECs were re-suspended in 500 μl PBS. In the meantime, the fluorescent intensities were assessed through adopting a flow cytometer (488 nm excitation, 521 nm emission).

**Transfection**

The vectors of si-NC and si-KLF2 were both amalgamated via GenePharma Co. (Shanghai, China). The cell transfections were conducted through exploiting Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) conforming to the manufacturer’s instruction.

**Statistical analysis**

Data from above diverse experiments are emerged as the mean ± SD. The statistical results were worked out through utilizing Graphpad statistical software (GraphPad Software Inc., San Diego, CA, USA). The unpaired two-tailed Student’s t-test and ANOVA were applied for computing the data in multiple groups. p Values <.05 was regarded as statistically significant.

**Results**

**H$_2$O$_2$ triggered oxidative stress injury of endothelial cells**

The effects of H$_2$O$_2$ on endothelial cells were first examined. The results in Figure 1(A) corroborated that cell viability was largely inhibited with the increase of H$_2$O$_2$ concentrations (50 and 100 μM, p < .05; 200 μM, p < .01; 300 μM, p < .001). At the same time, the cell apoptotic rate was appraised through flow cytometry, and the enhancement of cell apoptosis was...
discovered after H₂O₂ stimulation ($p < .001$, Figure 1(B)). In addition, the results from Western blot analysis showed that cell growth-related proteins (p53, p21, Bax, Cleaved-Caspase-9/-3) was enhanced by H₂O₂ stimulation ($p < .01$ and $p < .001$, Figure 1(C,D)). Besides that, the level of ROS was greatly elevated by H₂O₂ stimulation compared with the control group ($p < .01$, Figure 1(E)). Besides, HO-1 expression level was evidently restrained by H₂O₂ stimulation compared with the control group ($p < .01$, Figure 1(F)). These above results showed that H₂O₂ evoked obvious oxidative stress injury in endothelial cells.

**APS alleviates oxidative stress injury induced by H₂O₂**

H₂O₂ at 200 μM was selected as a proper working concentration in our following experiments. As shown in Figure 2(A),
suppressed cell viability triggered by H₂O₂ was elevated by APS at concentrations of 100 (p < .05), 200 (p < .01) and 300 μM (p < .05). Elevated cell apoptotic rate induced by H₂O₂ was suppressed by APS in HUVECs (p < .01, Figure 2(B)). Similarly, elevated expression of cell growth related proteins was also suppressed by APS (p < .01 and p < .001, Figure 2(C,D)). The increased level of ROS (Figure 2(E)) and repressed expression of HO-1 (Figure 2(F)) were both reversed by APS treatment (p < .01 and p < .05). These above results showed that APS alleviated H₂O₂-induced oxidative stress injury in endothelial cells.

**APS elevates the expression of KLF2 in endothelial cells**

In order to investigate the role of KLF2 in the protective effect of APS, the effect of APS on KLF2 expression was firstly investigated. As shown in Figure 3(A,B), the level of KLF2 was
increased by APS stimulation at concentrations of 100, 200 and 300 μM (p < .001) in the oxidative stress environment. Nrf2 is a crucial anti-oxidative target and KLF2 can enhance the antioxidant effect of Nrf2. Therefore, the relevancy between KLF2 and Nrf2 has been further uncovered. We observed that Nrf2 expression was evidently hindered by H2O2 stimulation (p < .001), meanwhile was enhanced by APS at concentrations of 200 (p < .05) and 300 μM (p < .01, Figure 3(C,D)). Other than this, when si-KLF2 was transfected into HUVECs, we discovered that enhancement of Nrf2 triggered by APS was obviously restrained in H2O2-stimulated endothelial cells (p < .001, Figure 3(E,F)). Thus, the above-involved results testified that APS was able to elevate KLF2 and Nrf2 expression in H2O2-stimulated endothelial cells. The above results also disclosed the positive relevancy between Nrf2 and KLF2 in H2O2 and APS co-administrated endothelial cells.

APS elevates KLF2 expression via the MEK/ERK signal pathway

The associated signal pathways were further investigated in endothelial cells after management with H2O2 and APS. As shown in Figure 5(A,B), H2O2 stimulation increased the rate of p/t-MEK and p/t-ERK to a certain degree (p < .01 and p < .001). In addition, APS stimulation induced more obvious elevation of the rate of p/t-MEK and p/t-ERK (p < .01 and p < .001). The specific MEK inhibitor SCH772984 was used in our experiment to inactivate the MEK/ERK signal pathway as shown in Figure 5(C). Further, we found that the elevating effect of APS on the expression of KLF2 was abolished by the combination of SCH772984 (p < .05, Figure 5(D)), identifying that APS elevated the expression of KLF2 via the MEK/ERK signal pathway.

Discussion

VECs are a highly differentiated monolayer of flat or polygonal cells covering the surface of the intima of the whole body, which lies between vascular wall tissues and blood [16,17]. The functions of VECs comprise selective permeation, inhibition of thrombosis, regulation of vascular tension, promotion of capillary formation and production of blood cell adhesion factors [18,19]. It is a multifunctional endocrine organ, which can secrete vasodilator, vasoconstrictor, growth regulator and cytokines [20]. The special structure and function of VECs are intimately linked to the occurrence of diversified vascular diseases, such as stroke, coronary heart disease, hypertension, diabetes [21–23]. Most of the traditional Chinese medicines contain polysaccharides, and the content is high. Accordingly, it is of great medical value to probe the protective effect of Polysaccharides from traditional Chinese medicine on VECs injury.

A substantial amount of evidence testified that reactive oxygen species (ROS) are important factors causing increase in oxidative stress in various diseases [24–26]. The increased level of ROS is able to induce apoptosis and anoikis, further leading to cell death [27–29]. In this study, we found that the levels of ROS induced by H2O2 were elevated by APS (p < .001, Figure 4(F)). APS alleviates oxidative stress injury through elevating KLF2

The expression of KLF2 was knocked down by specifically transfecting with si-KLF2 in endothelial cells and the transfection efficiency was shown in Figure 4(A,B) (p < .001). Then, we found that the accelerative function of APS in cell viability was abolished by repression of KLF2 (p < .001, Figure 4(C)). In the meantime, the suppressive impacts of APS on cell apoptosis and cell growth proteins were also weakened by the repression of KLF2 (p < .01, Figure 4(D,E)), respectively. In addition, the suppressed level of ROS triggered by APS was also elevated by the repression of KLF2 (p < .001, Figure 4(F)). Furthermore, the facilitative effect of APS on the expression of HO-1 was also annulled by the repression of KLF2 (p < .05, Figure 4(G,H)). Thus, the above results showed that APS alleviated oxidative stress injury through elevating KLF2.
endothelial dysfunction [24,25]. Moreover, the study has corroborated that, H₂O₂ can cause endothelial cell apoptosis and endothelial dysfunction through cell membranes [26]. As reported by the above research, we utilized H₂O₂ to make a cell damage model in endothelial cells. We discovered that H₂O₂ largely suppressed cell viability and obviously induced cell apoptosis. In addition, the enhancement of ROS level was found in H₂O₂-stimulated HUVECs. HO-1 can catalyze the degradation of heme, its products possess anti-oxidative damage, anti-inflammatory, anti-apoptotic and anti-proliferative functions [27]. Enhancement of HO-1 is universally regarded as a marker of oxidative stress, which has a protective impact on cell damage caused by oxidative stress [28]. In consonance with the aforementioned research, we also observed the repression of HO-1 in H₂O₂-managed HUVECs. Thus, suppressed expression of HO-1 in H₂O₂ stimulated HUVECs might be a protective mechanism of antioxidant stress in cells.

APS is a sort of effective components of Astragalus membranaceus, which has a wide range of pharmacological effects [29]. At present, most researches about APS focus on the protection of cardiac myocytes, and the anti-oxidant effect of APS has been reported. Chen et al. testified that APS could protect cardiac stem and progenitor cells through restraining oxidative stress-regulated apoptosis in diabetic hearts [30]. Han et al. also reported that APS could protect HUVECs against injury triggered by H₂O₂ through accelerating the cell antioxidant capacity and NO bioavailability [31]. In our present study, we observed that APS treatment effectively rescued H₂O₂ evoked cell viability inhibition. Then, APS also repressed H₂O₂ induced cell apoptosis and ROS level. Besides that, the expression of HO-1 was effectively ascended by APS treatment. Thus, we concluded that APS treatment effectively alleviated H₂O₂-induced cell injury.

A large number of studies have corroborated that vascular endothelial dysfunction can directly lead to cardiovascular diseases such as atherosclerosis and thrombosis [32,33]. KLF2 is widely expressed in blood vessels, but its expression content varies in different parts. Besides that, KLF2 has an important protective effect on the biological function of...
vascular endothelium. For example, overexpressed KLF2 robustly triggered endothelial nitric oxide synthase expression and total enzymatic activity in umbilical vein endothelial cells, which has been regarded as a neoteric controller of endothelial activation in response to pro-inflammatory stimuli [34]. Oxidation plays a dominant role in oxidative stress, leading to the accumulation of ROS in the body and the occurrence of cytotoxicity. After oxidative stimulation, transcription factor Nrf2 can reduce ROS level and improve the viability of cells, moreover, overexpression of KLF2 can enhance the antioxidant effect of Nrf2 [35]. In our present study, we found that APS treatment obviously elevated KLF2 and Nrf2 expression. Moreover, there is a positive correlation between KLF2 and Nrf2. Further study found that the repression of KLF2 abolished the protective activity of APS on H2O2-triggered cell injury by suppressing cell viability, promoting cell apoptosis and ROS, meanwhile hindering HO-1 expression.

MEK/ERK is a momentous signal transduction pathway, which takes part in adjusting oxidative stress injury [36]. Polidoro et al. disclosed that vitamin D could protect human endothelial cells against H2O2-triggered oxidant injury via activation of MEK/ERK pathway [37]. Sun et al. reported that nerve growth factor (NGF) protected against oxygen and glucose deprivation-triggered oxidative stress and apoptosis by elevation of HO-1 via adjusting the MEK/ERK pathway [38]. We observed that MEK/ERK signal pathway related proteins of p-MEK and p-ERK were both elevated by H2O2 stimulation and more obvious elevation of p-MEK and p-ERK were found with APS management, identifying that APS treatment activated the MEK/ERK signal pathway in H2O2-injured HUVECs. In order to further demonstrate the relationship between MEK/ERK signal pathway and KLF2, the specific MEK/ERK pathway inhibitor SCH772984 was employed in our experiments. We found that the promoting effect of APS on KLF2 expression was abolished by the addition of SCH772984, identifying that APS elevated KLF2 expression through modulating MEK/ERK signal pathway.

Altogether, the present study showed that APS effectively alleviated H2O2-evoked cell injury in HUVECs by elevating KLF2 expression via modulating MEK/ERK signal pathway. These explorations provide novel therapeutic methods and targets for vascular injury-related diseases.

Disclosure statement
Authors declare that there is no conflict of interests.

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Figure 5. APS elevates KLF2 expression via modulating MEK/ERK signal pathway. (A–B) The MEK/ERK signal pathway associated proteins of p-MEK, t-MEK, p-ERK, t-ERK were detected through Western blot. (C) Specific MEK inhibitor SCH772984 was utilized and the effect of SCH772984 on the level of p-MEK, t-MEK, p-ERK, t-ERK were detected through Western blot. (D) KLF2 expression was detected through Western blot. All consequences were emerged as mean ± SD. N = 3, *p < .05, **p < .01, ***p < .001 vs. control group. #p < .05, ##p < .01, ###p < .001 vs. H2O2 group. $p < .01 vs. H2O2 + APS group.
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