Astragaloside IV Protects Against Oxidized Low-Density Lipoprotein (ox-LDL)-Induced Endothelial Cell Injury by Reducing Oxidative Stress and Inflammation

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Background: Endothelial injury is the main mechanism of atherosclerosis, and is caused by oxidized low-density lipoprotein (ox-LDL). Astragaloside IV (AS-IV) is the primary active ingredient of the Chinese herb Huangqi, and exhibits antioxidant and anti-inflammatory properties in cardiovascular diseases. This study investigated the protective effect of AS-IV in human umbilical vein endothelial cells (HUVECs).

Material/Methods: HUVEC cells were induced with ox-LDL to establish an in vitro atherosclerosis model. Then HUVECs were pretreated for 1 h with AS-IV at different concentrations (10, 20, and 50 μM) and then exposed to ox-LDL (100 μg/mL) for 48 h. The cell viability, lactate dehydrogenase (LDH) release, apoptosis, migration, intracellular reactive oxygen species (ROS), and NADPH oxidase activity of HUVECs were measured. qRT-PCR was performed to measure the mRNA expressions of Nrf2, HO-1, TNFα, and IL-6. Enzyme-linked immunosorbent assay (ELISA) was performed to measure the supernatant contents of TNFα and IL-6.

Results: Exposure of HUVECs to ox-LDL reduced cell viability and migration, induced apoptosis, and increased intracellular ROS production and NADPH oxidase. Pretreatment with AS-IV (10, 20, and 50 μM) significantly enhanced the cell viability and migration, suppressed LDH release, apoptosis, ROS production, and NADPH oxidase in HUVECs, in a concentration-dependent manner. The AS-IV (50 μM) alone did not show significant differences from control. AS-IV increased mRNA expressions of Nrf2 and HO-1 and decreased mRNA expressions of TNFα and IL-6 in the ox-LDL-HUEVC cells. Furthermore, AS-IV reduced supernatant contents of TNFα and IL-6.

Conclusions: Astragaloside IV prevents ox-LDL-induced endothelial cell injury by reducing apoptosis, oxidative stress, and inflammatory response.

MeSH Keywords: Atherosclerosis • Endothelial Cells • Inflammation • Oxidative Stress • Receptors, Oxidized LDL

Abbreviations: AGEs – advanced glycation end-products; AS-IV – Astragaloside IV; ELISA – enzyme-linked immunosorbent assay; CCK-8 – Cell Counting Kit-8; FBS – fetal bovine serum; HO-1 – heme oxygenase-1; HUVECs – human umbilical vein endothelial cells; IL-6 – interleukin-6; LDH – lactate dehydrogenase; Nrf2 – nuclear factor erythroid 2-related factor 2; ox-LDL – oxidized low-density lipoprotein; qRT-PCR – quantitative real-time polymerase chain reaction; ROS – reactive oxygen species; TNFα – tumor necrosis factor-α; VSMCs – vascular smooth muscle cells

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Background

Atherosclerosis is a chronic inflammatory disease of the arterial wall and is involved in the pathogenesis of many cardiovascular diseases, thus leading to accumulation of lipid plaques and aorta and coronary artery stenosis [1]. Endothelial injury and apoptosis are the main pathological processes of atherosclerosis, and trigger the thrombosis process and accelerates the formation of atherosclerotic plaque [2], thus increasing susceptibility to ischemic events and acute cardiovascular symptoms [3]. Endothelial injury can be caused by a variety of factors, such as oxidized low-density lipoprotein (ox-LDL), advanced glycation end-products (AGEs), and angiotensin [4]. Increased levels of reactive oxygen species (ROS), such as superoxide anion, when exceeding the antioxidant capacity, can induce oxidative stress and promote vascular wall LDL oxidation, resulting in a high level of ox-LDL, leading to apoptosis of vascular endothelial cells and subsequent endothelial dysfunction [5,6]. Endothelial dysfunction is the initial reversible step in the formation of atherosclerosis. Therefore, treating endothelial dysfunction has become an important therapeutic strategy for preventing or reversing atherosclerosis.

Astragaloside IV (AS-IV) is a major compound extracted from the plant Astragalus membranaceus and has recently been shown to have multiple pharmacological effects on the liver, nervous system, hematopoietic system, endocrine system, cardiac function, collagen metabolism, and organ immune system [7]. In addition, AS-IV has been shown to promote proliferation of human umbilical vein endothelial cells and the formation of tubular structures in vitro [8]. AS-IV also stimulates angiogenesis via phosphoinositide 3-kinase/Akt, STAT3, and ERK1/2 signaling pathways [9,10]. These observations suggest that AS-IV may have important effects on cardiovascular disease. However, the role of AS-IV in the cellular model of atherosclerosis is unclear and related mechanisms are still unknown.

In the present study, we constructed an in vitro atherosclerosis model by exposing endothelial cells to ox-LDL. Effects of AS-IV on ox-LDL-induced endothelial injury, apoptosis, migration, oxidative stress, and inflammation were investigated. We also quantitatively analyzed mRNA expressions of antioxidant and inflammatory pathways such as nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), tumor necrosis factor-α (TNFα), and interleukin-6 (IL-6). This study will add AS-IV as a potential candidate in the treatment of endothelial injury in atherosclerosis and other cardiovascular diseases.

Material and Methods

Reagents and chemicals

Human umbilical vein endothelial cells (HUVECs) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Astragaloside IV (purity >98%, Cat. No. 84687-43-4) was purchased from Nanjing Spring & Autumn Biological Engineering Co. (Nanjing, China), dissolved in DMSO, and frozen at –80°C. Ox-LDL (oxidized low-density lipoprotein) was purchased from Beijing Solarbio Life Science Company (Cat. No. H7950, Beijing, China). Low-glucose Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and AnnexinV-FITC kits were purchased from Invitrogen (Cat. No. V13241, Carlsbad, CA). Cell counting kit-8 (CCK-8) kits were obtained from Beyotime Institute of Biotechnology (Cat. No. C0037, Shanghai, China). LDH assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Cat. No. A020-1, Nanjing, China). The Transwell chambers (8-μm diameter, 24 wells) were purchased from Corning, Inc. (Corning, NY). Matrigel matrix was purchased from BD System (Cat. No. 356234, Franklin Lakes, NJ). DCFH-DA (2’,7’-dichlorofluorescin diacetate) was obtained from Sigma Chemical Co. (Cat. No. D6883, St. Louis, MO). NADPH oxidase kits were purchased from Genmed Scientific, Inc. (Cat. No. GM550067, Shanghai, China). ELISA kits for TNFα (Cat. No. 88-7346-77) and IL-6 (Cat. No. 88-7066-77) were purchased from Invitrogen (Carlsbad, CA).

Cell culture and treatment

Endothelial cell line HUVECs were cultured in DMEM (low-glucose) supplemented with 10% FBS, and maintained in a humidified atmosphere containing 5% CO₂ at 37°C. The medium was refreshed every 2–3 days after cells had reached confluence, and experiments were performed in cells at passages 3 to 8. HUVECs were pretreated for 1 h with AS-IV at different concentrations (10, 20, and 50 μM) and then exposed to ox-LDL (100 μg/mL) for 48 h. The control group received 0.1% DMSO as vehicle. Our pilot study found that this concentration of DMSO had no cytotoxic effect on the HUVEC cells in the presence or absence of ox-LDL.

Cell viability assay

The Cell Counting Kit-8 (CCK-8) was used to measure cell viability. HUVECs were cultured in 96-well plates (1×10⁴ cells/mL, 100 μL/well). HUVEC cells were pretreated with AS-IV (10, 20, and 50 μM) and then exposed to ox-LDL (100 μg/mL) for 48 h. After washing 3 times with PBS, CCK-8 solution (1:10 dilution) was added to the medium and incubated at 37°C for 1 h. The absorbance was measured at 450 nm by a microplate reader.
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**LDH release assay**

Lactate dehydrogenase (LDH) release assay was used to evaluate LDH content in culture medium to quantify the protective effect of AS-IV on endothelial cells from ox-LDL-induced injuries. HUVECs were cultured in 96-well plates at a density of 1×10⁴ per well (100 μL DMEM). After 48 h of various treatments, the LDH content in the media was assessed using an LDH activity kit according to the manufacturer’s instructions.

**Apoptosis assay**

Flow cytometry was applied to determine the percentage of apoptotic cells using the Annexin V-FITC kit following manufacturer's instructions. HUVECs were cultured in 24-well plates (2×10⁴ cells/well, 1 mL) and treated with AS-IV (10, 20, and 50 μM) for 1 h, followed by 48 h of ox-LDL exposure. Then, the HUVECs were harvested and washed 3 times with PBS and resuspended at a density of 5×10⁶ cells/mL. Then, cells were added to 5 μL of Annexin V-FITC and 5 μL of PI solution in 100 μL 1×binding buffer, and incubated for 15 min in the dark. The percentage of Annexin V-FITC-positive/PI-negative cells and Annexin V-FITC-positive/PI-positive cells was analyzed by flow cytometry (FACSCalibur, BD).

**Migration assay**

HUVECs were seeded into the upper chamber of Transwell chambers (8-μm pore size diameter and 24 wells) in 100 μL DMEM medium supplemented with 0.1% FBS at a density of 1×10⁵ cells/mL. The lower chamber contained 600 μL DMEM medium supplemented with 10% FBS. After 48 h incubation at 37°C of various treatments, cells in the upper chamber were wiped away a cotton swab. The migrated cells were fixed with 37°C of various treatments, cells in the upper chamber were fixed with 95% ethanol for 10 min at room temperature, and then stained with 0.1% crystal violet at 22°C for 30 min. The stained migrated cells were counted under a light microscope (magnification, ×100) from 10 fields to calculate the percentage of migrated cells.

**Measurement of intracellular ROS**

HUVECs were cultured in 24-well plates (2×10⁵ cells/well, 1 mL) and treated with AS-IV (10, 20, and 50 μM) for 1 h, followed by 48 h of ox-LDL exposure. Then, HUVECs were incubated with DCFH-DA (1: 1000 v/v) at 37°C for 45 min in the dark and by 48 h of ox-LDL exposure. Then, cells were harvested, protein was extracted, and NADPH oxidase activity was evaluated by use of an NADPH oxidase kit according to the manufacturer’s instruction [12].

**Enzyme-linked immunosorbent assay (ELISA)**

HUVECs were cultured in 24-well plates (2×10⁵ cells/well, 1 mL) and treated with AS-IV (10, 20, and 50 μM) for 1 h, followed by 48 h of ox-LDL exposure. Then, the cellular supernatant was collected. The levels of the proinflammatory cytokines of TNFα and interleukin-6 (IL-6) were analyzed using an ELISA kit, measuring optical density at 450 nm using a microplate reader (Rico RK201, Shenzhen Rico Technology Co., Shenzhen, Guangdong, China). All ELISA assays were performed 3 times, according to the manufacturer’s instructions. The serum TNFα and IL-6 concentrations of each group was determined according to the standard curve established by recombinant TNFα and IL-6, respectively.

**Quantitative real-time PCR**

Total RNA was extracted from HUVEC cells of different groups with Trizol to obtain cDNA by reverse transcription. qRT-PCR was performed to determine the expression levels of Nrf2, HO-1, TNFα, and IL-6 using SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA) in an ABI PRISM 7500 Fast Real-time PCR instrument (Applied Biosystems, Foster City, CA). Amplification conditions were set as follows: pre-denaturation at 95°C for 2 min, then 40 cycles of 95°C for 20 s and 58°C for 20 s. Human GAPDH gene was used as an internal reference. Specific primers were shown below: Nrf2: forward, 5'-TCC GGG TGT GTT GTT TGC AA-3', reverse, 5'-CGG CGG CGA GAT AAA GAG TT-3'; TNFα- forward, 5'-CTT GCC ACT TG-3', reverse, 5'-TGC GCC GTA AAT GGT GGT CT-3'; IL-6 forward, 5'-GAG GCC ACG ACC TTC TGG TAT G-3', reverse, 5'-CAC GCC GAT TGT CTC TCC-3'; GAPDH forward, 5'-CAT CCA ACA TTA GGT GGT CT-3', reverse, 5'-TCC AAAG CGG CTG GCC-3'; GAPDH: forward, 5'-TCT GAA CCT TCC AAA GAT GGC-3', reverse, 5'-TGC GCT AGG CAA GTC TTC TCA-3'; GAPDH: forward, 5'-CCT GAT CAT CAGCAA TG-3', reverse, 5'-CCA TCC ACA GTC TTC TGG GT-3'. The data was analyzed according to the 2−ΔΔCT method. All reactions were performed at least 3 times.

**Statistical analysis**

Values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 19.0 software (IBM, USA). Differences between groups were calculated using analysis of variance (ANOVA), followed by the Student-Newman-Keuls (SNK) test. Power analysis of the difference in cell viability was performed. The parameters needed for the...
to measure apoptotic rate. ox-LDL significantly increased the release in HUVECs. Annexin V-FITC/PI staining was performed alone (50 μM) did not significantly influence cell viability or LDH release in HUVECs. However, AS-IV (10, 20, and 50 μM) significantly attenuated the decrease in cell viability and increase in LDH release in HUVEC cells with ox-LDL, in a concentration-dependent manner. The pretreatment with ox-LDL (100 μg/mL) significantly reduced cell viability and enhanced LDH release in HUVEC cells, compared with normal control (P<0.05) (Figure 1A, 1B). The pretreatment with AS-IV prevented ox-LDL-induced endothelial injury (Figure 1C, 1D).

**Results**

**AS-IV prevented ox-LDL-induced endothelial injury**

Cell viability was performed to assess the protective effect of AS-IV on endothelial injuries induced by ox-LDL. After 48-h exposure, ox-LDL (100 μg/mL) significantly reduced cell viability and enhanced LDH release in HUVEC cells, compared with normal control (P<0.05) (Figure 1A, 1B). The pretreatment with AS-IV (10, 20, and 50 μM) significantly attenuated the decrease in cell viability and increase in LDH release in HUVEC cells with ox-LDL, in a concentration-dependent manner. However, AS-IV alone (50 μM) did not significantly influence cell viability or LDH release in HUVECs. Annexin V-FITC/PI staining was performed to measure apoptotic rate. ox-LDL significantly increased the apoptotic rate at 48 h, and AS-IV (at 10, 20, and 50 μM) significantly attenuated the apoptotic rate induced by ox-LDL (Figure 1C, 1D).

**AS-IV inhibited ox-LDL-induced migration in HUVEC cells**

To explore the migration ability of ox-LDL-induced HUVECs by AS-IV, we performed Transwell assay. Cells were treated with ox-LDL (100 μg/mL) or ox-LDL plus various concentrations of AS-IV (10, 20, and 50 μM) for 48 h, then the migrated cells were stained with crystal violet. Representative images showed that, compared with control group (Figure 2A), the ox-LDL group showed fewer stained cells (Figure 2B). AS-IV treatment increased the number of stained cells at 10 μM (Figure 2C), 20 μM (Figure 2D), and 50 μM (Figure 2E). Quantitative analysis showed AS-IV treatment significantly increased the number of migrated HUVEC cells exposed to ox-LDL, in a concentration-dependent manner (P<0.05) (Figure 2F).
AS-IV inhibited ROS production and activated the Nrf2-HO-1 pathway in ox-LDL-induced HUVEC cells

To investigate the effect of AS-IV on ROS production, we measured intracellular ROS by fluorescent probe DCFH-DA and NADPH oxidase activity by colorimetric analysis. ox-LDL significantly increased the intracellular ROS (Figure 3A) and NADPH oxidase activity (Figure 3B) of HUVEC cells. However, AS-IV decreased the intracellular ROS and NADPH oxidase activity in ox-LDL-induced HUVEC cells (P<0.05). Our results show that ox-LDL promoted oxidative stress and AS-IV exerted an antioxidant effect on ox-LDL-induced HUVEC cells. To explore the molecular mechanism of AS-IV in oxidative stress, we then performed qRT-PCR to measure the mRNA levels of 2 antioxidant
genes: Nrf2 and HO-1. After exposure to ox-LDL, the mRNA expressions of Nrf2 and HO-1 were significantly decreased. Treatment with AS-IV significantly increased Nrf2 and HO-1 mRNA expressions in a concentration-dependent manner (P<0.05) (Figure 3C, 3D).

AS-IV inhibited inflammatory response in ox-LDL-induced HUVEC cells

To determine whether AS-IV regulates inflammatory response in ox-LDL-induced HUVEC cells, we measured TNFα and IL-6 contents in supernatant media of cultured cells by use of ELISA. ox-LDL significantly increased the supernatant levels of TNFα and IL-6 in HUVEC cells, which was significantly attenuated by AS-IV, in a concentration-dependent manner (P<0.05) (Figure 4A, 4B). We further performed qRT-PCR to measure the mRNA levels. The mRNA expressions of TNFα and IL-6 were significantly decreased after exposure to ox-LDL, and were significantly increased by AS-IV treatment, in a concentration-dependent manner (P<0.05) (Figure 4C, 4D).

Discussion

In this study we evaluated the effect of AS-IV on ox-LDL-induced HUVECs injury. ox-LDL (100 μg/mL) exposure reduced cell viability and increased LDH release in supernatant media, which was reversed by treatment with AS-IV (10, 20, and 50 μM) for 48 h. In HUVEC cells exposed to ox-LDL, AS-IV reduced apoptosis, promoted cell migration, and suppressed oxidative stress and inflammatory cytokine production. The mechanisms might be related to increased mRNA expression of Nrf2 and HO-1 and decreased mRNA expression of TNFα and IL-6. Therefore, AS-IV protects HUVEC cells against ox-LDL-induced injury.
injury through modulation of anti-oxidative and anti-inflammatory response pathways.

Atherosclerosis is a contributing factor of many cardiac and cerebral vascular diseases, and can be initiated and promoted by several important processes, include oxidative stress [13], vascular endothelial injury [14], and release of inflammatory mediators [15]. Ox-LDL contributes to the formation and progression of atherosclerotic plaque through several mechanisms, including the induction of endothelial injury, endothelial apoptosis, foam cell formation from macrophages, and disruption of antioxidant capabilities [16]. Therefore, we used ox-LDL as an agent to induce endothelial injury of HUVECs as an in vitro model to investigate the protective effect of AS-IV.

The present study shows that AS-IV promoted proliferation and migration of HUVECs exposed to ox-LDL. Endothelial cells play key roles in maintaining physiological function of the cardiovascular system through regulating blood circulation, coagulation, angiogenesis, and inflammatory responses, and proliferation and migration of endothelial cells contribute to angiogenesis and vessel sprouting [17]. Our study shows that AS-IV protects against endothelial injury in prevention and therapy of atherosclerosis, which is in accordance with an in vivo study that reported AS-IV inhibited atherosclerotic lesions in hyperlipidemia-induced atherosclerotic ApoE(−/−) mice [18]. However, our results are also contradictory to previous reports on regulation of AS-IV in endothelial proliferation and migration. For example, AS-IV inhibited proliferation and migration of vascular smooth muscle cells (VSMCs) stimulated by platelet-derived growth factor-BB and Angiotensin II, respectively [19,20]. This inconsistency can be explained by the different roles in atherosclerosis of 2 major type of cells in blood vessel walls: endothelial cells and VSMCs. The proliferation and migration of VSMCs promote atherosclerosis development [21]. Therefore, our study was supported by another study reporting that AS-IV exerts pro-angiogenic effects through stimulating the proliferation and migration of HUVECs [22].

Figure 4. Inhibitory effect of AS-IV on inflammatory response of ox-LDL-induced HUVECs. After pretreated with AS-IV (10, 20, and 50 μM) for 1 h, cells were exposed to ox-LDL (100 μg/mL) and incubated for 48 h. Then, cells and supernatant media were collected. ELISA was performed to determine the production of proinflammatory cytokines, and showed AS-IV decreases TNFα (A) and IL-6 (B) contents in supernatant media of ox-LDL-induced HUVECs. qRT-PCR shows that ox-LDL reduced mRNA levels of the TNFα (C) and IL-6 (D), which was attenuated by AS-IV. *** P<0.001 vs. control; * P<0.05; ** P<0.01, ### P<0.001 vs. ox-LDL.

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This study showed that AS-IV suppressed oxidative stress and activated the Nrf2 antioxidant pathway in the protective effect on ox-LDL-induced endothelial injury. Oxidative stress is induced by excessive ROS, and acts as a critical mechanism in vascular homeostasis and atherosclerosis [13]. Intracellular ROS production promotes endothelial cell apoptosis in the development of atherosclerosis [23], while NADPH oxidase can generate vascular ROS and is involved in endothelial apoptosis [24]. Uncontrolled production of ROS leads to vascular injury, which is in accordance with our results showing that ox-LDL enhanced production of ROS and NADPH oxidase activity, with reduced cell viability. Nrf2 is an endogenous antioxidant defense system that is activated under stress conditions, thereby maintaining cellular redox homeostasis. Activation of the Nrf2/HO-1 signaling pathway protects endothelial cells from ox-LDL-induced injury [25]. Our results showed that ox-LDL promoted oxidative stress and decreased the mRNA expression of Nrf2, which is inconsistent with previous reports. In human umbilical vein endothelial cells, ox-LDL treatment increased Nrf2 protein expression [26] and nuclear Nrf2 translocation [27]. This contradiction can be explained by the fact that ox-LDL can induce endothelial senescence [28], accompanied by increased NADPH oxidases and NADPH oxidase-derived ROS [29]. In HUVEC, endothelial senescence was caused by suppressed expression of Nrf2 at the transcriptional level [30], and this decreased expression was modulated by direct targeting of Nrf2 mRNA by several microRNAs (miRNAs) [31,32]. AS-IV demonstrates a broad range of anti-oxidative stress by activating the Nrf2 antioxidant pathway, thereby protecting cortical neurons against ischemia/reperfusion injury [33], inhibiting hepatic stellate cell activation [34], and protecting lipopolysaccharide-induced microglia [35]. In particular, activation of Nrf2 by AS-IV protects the integrity of the blood-brain barrier, which was destroyed by LPS [36]. Since endothelial barrier disruption is an important mechanism by which macrophage-derived foam cells promote the development of atherosclerosis [37], Nrf2 activation may be an important mechanism of AS-IV against atherosclerosis, but this has not been reported in the literature. In addition, the potential regulation of AS-IV on cytoplasmic and nuclear Nrf2 protein and endothelial senescence-associated microRNAs deserves further investigation.

This study showed that AS-IV suppressed inflammatory response, as evidenced by decreased proinflammatory cytokines

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Conclusions

The present study provides evidence that astragaloside IV treatment can protect against ox-LDL-induced endothelial injury, with suppressed oxidative stress and inflammatory response, which is related to enhanced expression of Nrf2 and HO-1 signaling pathways. These findings suggest that astragaloside IV has clinical potential in prevention of atherosclerosis.

Conflict of interest

None.
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