

Advanced oxidation protein products induce the apoptosis of vascular endothelial cells via activating Nrf2 pathway in atherosclerosis.

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Abstract

Objective: To explore the influence of Advanced Oxidation Protein Products (AOPPs) on vascular endothelial cells proliferation and apoptosis and the role of Nrf2 pathway in the process.

Methods: Different concentrations of AOPPs were used to treat vascular endothelial cells (HUVEC) for 12, 24 and 48 h. Vascular endothelial cells were treated with high, middle and low concentrations of AOPPs for 24 h and flow cytometry was used to determine the changes of cell cycle, apoptosis and reactive oxygen species. Western blotting was used to determine the protein expression of Caspase 3, Caspase 8, Caspase 9, Nrf2 and its downstream gene *HO-1*.

Results: The vascular endothelial cell viability decreased gradually with the increase of AOPPs concentrations from 0 ug/ml to 50 ug/ml, and then further decreased with the increase of concentrations from 50 ug/ml to 200 ug/ml, and the time had no influence on cell viability. Therefore, concentrations of 2, 50 and 200 ug/mL and time of 24 h were used for the further study. Vascular endothelial cells mainly focused at G1 stage at high concentration of AOPPs. The number of apoptotic cells, as well as reactive oxygen and expressions of Caspase 3, Caspase 8, Caspase 9, Nrf2 and *HO-1*, increased with the increase of AOPPs concentrations.

Conclusion: In atherosclerosis, AOPPs could induce the apoptosis of vascular endothelial cells and inhibit the proliferation by activating Nrf2 pathway.

Keywords: Atherosclerosis, Advanced oxidation protein products, Nrf2, Apoptosis.

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Introduction

Atherosclerosis is nowadays generally accepted as an inflammatory disease. It is known that local inflammation occurs during the formation of the plaques, as macrophages and other immuno-competent cells are present in the lesions from an early stage [1,2]. Recent advances in basic science have established a fundamental role for inflammation in mediating all stages of this disease [3]. The early phase of atherosclerosis involves the recruitment of inflammatory cells from the circulation and their transendothelial migration [4].

Endothelial cells have long been viewed as a passive lining of blood vessels with negative properties such as nonreactive to blood components. They play a critical role in thromboregulation by virtue of a surface-connected fibrinolytic system [5,6]. They produced a Neutrophil Chemotactic Factor (NCF) upon stimulation with Tumor Necrosis Factor-alpha (TNF-alpha), Interleukin-1 beta (IL-1 beta), or Lipopolysaccharide (LPS) [7]. Vascular endothelial cells undergo morphogenesis into capillary networks in response to

angiogenic factors [8]. The abnormal proliferation of vascular endothelial cells was closely related with atherosclerosis. Advanced Oxidation Protein Products (AOPPs) are new protein markers of oxidative stress with pro-inflammatory properties [9]. They are derived from oxidation-modified albumin (its aggregates or fragments), but also of fibrinogen, and lipoproteins [10]. The accumulation of AOPPs has been linked to vascular lesions in diabetes, chronic renal insufficiency, and atherosclerosis [11]. Increased level of plasma AOPPs has been found in patients with uremia and nonuremic subjects with coronary artery disease [12]. During the oxidation process, several proteins have attracted scholars' interest. Transcription factor Nuclear factor erythroid 2 p45 related factor 2 (Nrf2) is a major regulator of genes encoding phase 2 detoxifying enzymes and antioxidant stress proteins in response to electrophilic agents and oxidative stress [13,14]. It regulates antioxidant-responsive element-mediated induction of cytoprotective genes in response to oxidative stress. Heme oxygenase-1 (*HO-1*) is a stress-inducible rate-limiting enzyme in heme degradation. Studies show that it confers

cytoprotection against oxidative injury and provides a vital function in maintaining tissue homeostasis [15]. Though there are lots of studies focusing on mechanisms of apoptosis of vascular endothelial cells, few of them studied the role of advanced oxidation proteins in the process and its relationship with Nrf2 pathway. In this study, we aimed to study the effect of AOPPs on the proliferation and apoptosis of vascular endothelial cells in atherosclerosis and the role of Nrf2 pathway in the process.

Material and Methods

Cell culture

Vascular Endothelial Cells (HUVEC) were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were routinely cultured in RPMI-1640 supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified cell incubator with an atmosphere of 5% CO₂ at 37°C. Cells growing at an exponential rate were used for the experiments.

Influence of AOPPs on vascular endothelial cell proliferation

Vascular endothelial cell lines were treated with different concentrations of AOPPs (0, 1, 2, 5, 10, 20, 50, 100 and 200 µg/ml) for 12, 24 and 48 h, respectively. For proliferation assays, cells were seeded in 96-well plates at 4,000 cells/well using Lipofectamine 2000 Reagent. Cell proliferation was measured using the Cell Counting Kit-8 assay (CCK-8, Boster, Wuhan, China). The proliferation analysis of each group in each individual experiment and the whole experiment process were performed in triplicate.

Flow cytometry

HUVEC cells were treated with 0, 50 and 200 µg/ml AOPPs for 24 h, and then cell cycle, cell apoptosis and reactive oxygen were determined by flow cytometry. Cell lines were selected and plated in a 96-well plate at a density of 2×10^3 cells/well in supplemented RPMI 1640 and incubated for 16 h before the cells were subjected to treatment in triplicate wells. After treatment, the cells were washed twice in phosphate-buffered saline (PBS) (2.68 mM KCl, 1.47 mM KH₂PO₄, 8 mM Na₂KPO₄, 136.75 mM NaCl) and counted. Fifty to one hundred thousand cells were selected and centrifuged 5 min at 1000 r/min. Annexin V-FITC mixed liquor of 195 µL was added to resuspend cytotrophoblast cells and 5 µL was added to mix. Centrifugation at 1000 r/min for 5 min was performed after cultivation 10 min. Sample was obtained after discarding supernatant and 10 µL Propidium Iodide (PI) was added. 2', 7'-Dichlorofluorescein diacetate was used as fluorescent probe to analyse the level of Reactive Oxygen Species (ROS). Afterwards, the sample was stilled in dark for 30 min. Finally, the apoptosis, cycle and reactive oxygen were detected using Flow Cytometry (FCM) on the Moflo (Dako Cytomation, Glostrup, Denmark).

Western blotting

To determine the protein expression of apoptosis proteins Caspase 3, Caspase 8, Caspase 9, and Nrf2 and its downstream gene *HO-1*, western blot assay was carried out. Total proteins were prepared from cell lines using Radioimmunoprecipitation Assay (RIPA) lysis buffer (Sigma, USA) supplemented with protease inhibitors. An equal amount (50 µg) of cellular lysates was separated on 10% SDS-Polyacrylamide Gel Electrophoresis (PAGE) minigels and transferred to nitrocellulose filter membranes (Hybond, Escondido, CA, USA). The membrane was blocked with Tris-Buffered Saline Tween-20 (TBST) containing 5% skimmed milk powder for 1 h at room temperature, followed by incubation in TBST containing 5% BSA (Sigma, St. Louis, MO, USA) and primary antibodies overnight at 4°C. Primary antibodies were detected using a peroxidase-coupled goat anti-rabbit secondary antibody (1:8000, ZSBio, Beijing, China) and EZ-ECL chemiluminescence Detection kit for HRP (Biological Industries, Beit-Haemek, Israel). The following primary antibodies were used: rabbit mAb Caspase 3, rabbit mAb Caspase 8, rabbit Caspase mAb 9, rabbit mAb Nrf2, rabbit mAb *HO-1* (Cell Signalling Technology, Danvers, MA, USA) and rabbit pAb GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Statistical analysis

Statistical analysis was performed using SPSS 18.0. Data are expressed as the mean ± Standard Error of the Mean (SEM) of three independent experiments. Multiple group comparisons were analysed using ANOVA with a post hoc test. P<0.05 was considered to indicate a statistically significant result.

Results

AOPPs increased vascular endothelial cell viability

To explore the effect of AOPPs on vascular endothelial cell proliferation, vascular endothelial cells were treated with 0, 1, 2, 5, 10, 20, 50, 100 and 200 µg/ml AOPPs for 12, 24 and 48 h, respectively. Results were shown in Figure 1. As shown, cell viability decreased with the increase of AOPPs concentrations from 0 µg/ml to 200 µg/ml AOPPs at 12 h, 24 h and 48 h. Moreover, the cell viability under concentrations of 2, 5, 10, 20, 50, 100 and 200 µg/ml was significantly lower than the control group A OPPs.

AOPPs increased cell apoptosis

Vascular endothelial cells were treated with 0, 2, 50 and 200 µg/ml AOPPs for 24 h and then cell cycle, cell apoptosis and reactive oxygen situation of vascular endothelial cells were determined by flow cytometry. As shown in Figure 2A, the G1 stage extended with AOPPs concentration increased gradually from 0 µg/ml to 200 µg/ml. The number of apoptotic cells increased with the increase of AOPPs concentration, and the value (Figure 2B). As shown in Figure 2C, fluorescence value

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for reactive oxygen increased with the increase of AOPPs concentration.

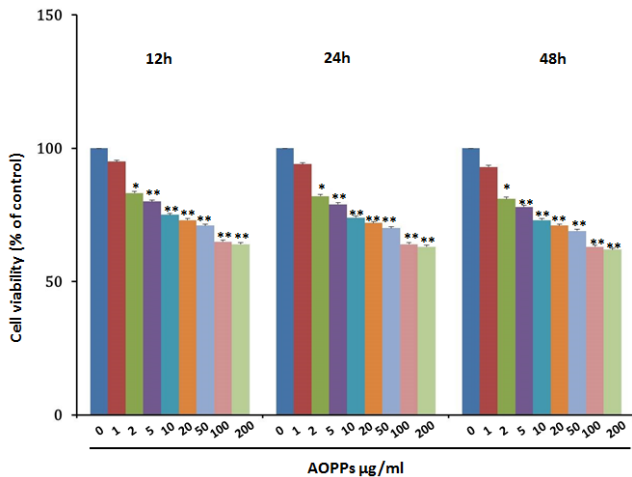


Figure 1. The cell viability of vascular endothelial cells changed after treated with 0, 0, 1, 2, 5, 10, 20, 50, 100 and 200 µg/ml AOPPs for 12, 24 and 48 h, respectively. * $P < 0.05$, ** $P < 0.01$ compared with cell viability at 0 µg/ml AOPPs, the value at 2 µg/ml AOPPs had significant difference.

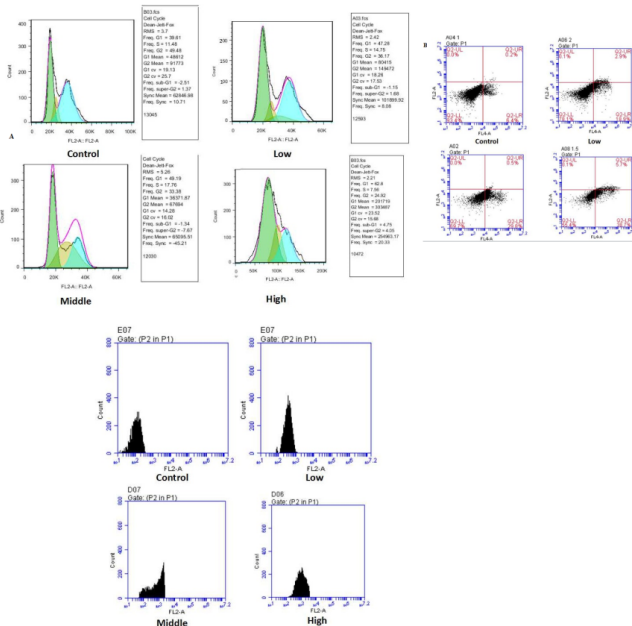


Figure 2. The cell cycle (A), cell apoptosis (B) and reactive oxygen (C) situation of vascular endothelial cells after treating with low, middle and high concentration of AOPPs for 24 h compared with the control.

AOPPs increased the level of apoptotic proteins

To explore the molecular mechanism on how AOPPs promote the apoptosis of vascular endothelial cells, the protein expression values of apoptotic protein Caspase 3, Caspase 8 and Caspase 9 were determined by western blotting. As shown in Figure 3, the protein expression of Caspase 3, Caspase 8 and Caspase 9 increased with AOPPs concentration increased from

0 µg/ml to 200 µg/ml. The value in AOPPs concentration of 2 µg/ml was almost the same with that in the control (0 µg/ml).

AOPPs increased the level of Nrf2

In this study, we determined the protein expression values of Nrf2 and its downstream gene *HO-1* by western blotting. Results showed the levels of Nrf2 and *HO-1* increased with the increase of AOPPs concentrations (Figure 4).

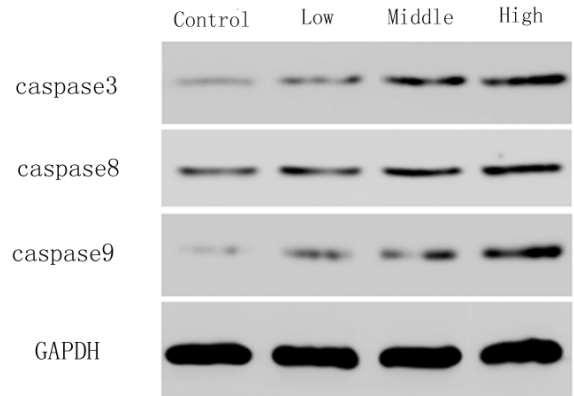


Figure 3. The protein expression values of Caspase 3, Caspase 8 and Caspase 9 in vascular endothelial cells after treating with low, middle and high concentration of AOPPs for 24 h compared with the control.

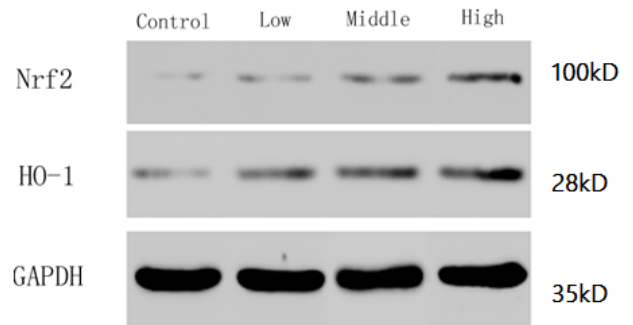


Figure 4. The protein expression values of Nrf2 and *HO-1* in vascular endothelial cells after treating with low, middle and high concentration of AOPPs for 24 h compared with the control.

Discussion

Atherosclerosis, the principal cause of heart attack, stroke and gangrene of the extremities, is responsible for 50% of all mortality in the USA, Europe and Japan [16,17]. It affects the major elastic and muscular arteries, but some vessels are largely spared while others may be markedly diseased. Atherosclerosis has long been recognized as having an inflammatory component, and this has a particularly important bearing on to its clinical complications as it may result in plaque instability [18]. It is observed in embryonic and injured tissue and is particularly prominent in the vicinity of solid tumors 1, involves the migration and proliferation of capillary endothelial cells [19]. Oxidative stress and expression of the Vascular Cell Adhesion Molecule-1 (VCAM-1) on vascular endothelial cells are early features in the pathogenesis of atherosclerosis and other inflammatory diseases [20].

Endothelial cell damage is one important pathophysiological step of atherosclerosis and restenosis after angioplasty [21]. AOPPs are new protein markers of oxidative stress with pro-inflammatory properties, accumulated in many pathological conditions [10].

In this study, we aimed to explore the effect of AOPPs on the proliferation of vascular endothelial cells and the related molecular mechanism. First, vascular endothelial cells were treated with 0, 1, 2, 5, 10, 20, 50, 100 and 200 µg/ml AOPPs for 12, 24 and 48 h, respectively, and cell viability was determined. Results showed cell viability decreased with the increase of AOPPs concentrations from 0 µg/ml to 200 µg/ml. Previous studies showed that AOPPs could induce the apoptosis of islet microvascular endothelial cells and enhanced the ROS level via a RAGE-mediated signalling pathway [22,23]. The present study showed similar effect of AOPPs on vascular endothelial cells. Then, effects of AOPPs on cell apoptosis and reactive oxygen were further studied. Concentrations of 2 µg/mL, 50 µg/ml and 200 µg/ml were selected as low, middle and high concentrations of AOPPs for further study. Vascular endothelial cells were then treated with 0, 2, 50 and 200 µg/ml AOPPs for 24 h, and then cell cycle, cell apoptosis and reactive oxygen situation of vascular endothelial cells were determined by flow cytometry. Results showed the cell cycle was mainly focused on G1 stage at high concentration of AOPPs, the number of apoptotic cells was significantly higher in high concentration of AOPPs compared with that in low and middle concentration of AOPPs and the control. It indicated high concentration of AOPPs can promote the apoptosis of vascular endothelial cells. Similar results are found in other studies, which indicate that AOPPs can also induce apoptosis in several kinds of cells like podocyte [24], intestine epithelial cells [25], and chondrocyte [26]. At last we explored the molecular mechanism about how AOPPs promote the apoptosis of vascular endothelial cells, the protein expression values of Caspase 3, Caspase 8, Caspase 9, Nrf2 and *HO-1* were determined by western blotting. Results showed the levels of Caspase 3, Caspase 8, Caspase 9, Nrf2 and *HO-1* at high concentration of AOPPs were significantly higher than those at low and middle concentration of AOPPs and the control. The levels increased with the increase of AOPPs concentration. Caspases are crucial mediators of programmed cell death (apoptosis). Among them, caspase-3 is a frequently activated death protease, catalysing the specific cleavage of many key cellular proteins [27]. Activated caspase-3 was detected in atherosclerotic [28]. Caspase 8 is a cysteine protease regulated in both a death-receptor-dependent and independent manner during apoptosis [29]. Caspase-9 is a member of caspase family of cysteine proteases that have been implicated in apoptosis and cytokine processing [30]. Caspase-8 and caspase-9 are considered to be two initiator caspases for mediating apoptotic signals from death receptors or from cytochrome c translocation, leading to activation of caspase-3 as a key factor in apoptotic cell death [31]. Transcription factor Nrf2 is a major regulator of genes encoding phase 2 detoxifying enzymes and antioxidant stress proteins which regulates the basal and inducible expression of

numerous detoxifying and antioxidant genes [32]. HO-1 is a stress protein induced in response to a variety of oxidative challenges [33]. The Nrf2/*HO-1* pathway is reported to be involved in cell death in many diseases [34,35], including some endothelial cells [36]. However, relationship between AOPPs and Nrf2 pathway in apoptosis of vascular endothelial cells has not been reported yet.

In conclusion, in the present study, we investigated role of AOPPs in proliferation and cell apoptosis of vascular endothelial cells and demonstrated that AOPPs could promote the apoptosis of vascular endothelial cells by activating Nrf2/*HO-1* pathway in atherosclerosis.

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