Original Paper

Alpha-Lipoic Acid Protects Human Aortic Endothelial Cells Against H₂O₂-Induced Injury and Inhibits Atherosclerosis in Ovariectomized Low Density Lipoprotein Receptor Knock-Out Mice

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Key Words

Atherosclerosis • Alpha-lipoic acid • Estrogen receptor • Oxidative injury

Abstract

Background/Aims: Diseases caused by atherosclerosis are the leading causes of death in postmenopausal women, owing to the loss of estradiol. Hormone replacement therapy (HRT) provides short-term beneficial effects in the treatment of cardiovascular disease for postmenopausal women but may increase the risk of stroke and gynecological cancer. Therefore, a substitute for HRT is urgently needed. **Methods:** In this study, we examined the effectiveness of alpha-lipoic acid (ALA), a natural potent antioxidant, in preventing the development and progression of atherosclerosis in the low density lipoprotein receptor deficient (*Ldlr*^{-/-}) mouse model, using western blot analysis, immunohistochemistry, Oil-red-O, elastin staining and TUNEL assay. We also examined the protective effect of ALA in human aortic endothelial cells (HAECs) against H₂O₂-induced oxidative injury, using western blotting, immunofluorescence staining, and monocyte adhesion assay. **Results:** We showed that ALA treatment significantly reduced the atherosclerosis induced by ovariectomy and high fat diet in the *Ldlr*^{-/-} mouse model and restored expression of estrogen receptors (ER α and ER β), which reduced the progression of atherosclerosis. Moreover, ALA treatment attenuated monocyte adhesion, suppressed cellular apoptosis, and eliminated excessive generation of intracellular reactive oxygen species (ROS) by reducing the protein levels of ROS-generating enzymes Nox4 and p22phox, as well as inhibiting NF- κ B activation in HAECs stimulated by H₂O₂. **Conclusions:** ALA could provide a potential treatment for atherosclerosis in postmenopausal patients.

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Introduction

Despite considerable therapeutic advances over the past few decades, atherosclerosis remains a leading cause of death worldwide, especially among postmenopausal women [1, 2]. Compared with premenopausal women, postmenopausal women have a higher incidence of cardiovascular disease due to loss of responsiveness to either internal or even externally provided estrogen [3]. It has been postulated that estrogen deficiency contributes to the high morbidity of atherosclerosis in postmenopausal women through a role in numerous atherogenic processes, including inflammation [4, 5], vascular smooth muscle cell proliferation [6], generation of reactive oxygen species (ROS) [7], and apoptosis of vascular endothelial cells [8]. Some studies have demonstrated that hormone replacement therapy (HRT) can lower the risk of cardiovascular disease in postmenopausal women, but only when therapy is initiated in the early stages after menopause [9-12].

Many studies have shown that inflammation and oxidative stress play a central role in the progression of atherosclerosis [2, 13, 14]. Oxidative stress, as a result of excessive generation of intracellular ROS including hydroxyl radicals (OH⁻), hydrogen peroxide (H₂O₂), and superoxide anion (O₂⁻), is reported to play a key role in the development and progression of atherosclerosis via promoting endothelial dysfunction, inflammation, and lipid peroxidation [15, 16]. The NADPH oxidase (Nox) family of enzymes and their regulatory subunits, including p22phox, p47phox, Noxa1, and p67phox, are important sources of ROS in the vasculature triggered by stress, hormones, and vasoactive agents. ROS is reported to activate NF-κB [17], which plays a key role in various inflammatory and immune responses and is linked to the development of atherosclerosis [18]. In addition, it has been shown that NF-κB is involved in the transcriptional regulation of Nox subunit expression [19, 20]. Therefore, pharmacological agents that could attenuate inflammation and oxidative stress by interrupting the NF-κB pathway should possess anti-atherogenic effects.

Estrogen is directly or indirectly involved in regulating immune responses, cell proliferation, inflammation, and cell adhesion [18]. Estrogen signaling has been shown to reduce the development of atherosclerosis through rapid non-genomic effects and/or the classic pathway by binding to estrogen receptors (ERα and ERβ) [21-23]. Estrogen also has antioxidant properties by inhibiting the expression of Nox and the generation of O₂⁻ superoxide anion [24]. Furthermore, estrogen exerts its atheroprotective effects by binding to its receptors to generate nitrogen monoxide in endothelial cells, which tends to have a protective function on blood vessels [25-27]. Unfortunately, the expression levels of estrogen receptors decline during the late stage of menopause. As a result, estrogen supplementation at this stage cannot reverse the risk of atherosclerosis [28, 29]. Therefore, a pharmacological agent that could promote the levels of estrogen receptors would benefit postmenopausal women by protecting against atherosclerosis.

Alpha-lipoic acid (ALA), also known as 1, 2-dithiolane-3-pentanoic acid, 1, 2-dithiolane-3-valeric acid, or thioctic acid, is reported to be a potent antioxidant that can eliminate intracellular ROS [30-32]. Several lines of evidence have shown that ALA has anti-inflammatory and anti-obesity effects against the pathological process of atherosclerosis [33-35]. However, the mechanisms of the protective function of ALA and its effects on the development of postmenopausal atherosclerosis remain unknown.

Materials and Methods

Materials

Human aortic endothelial cells (HAECs) were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cell culture medium (DMEM, RPMI-1640) and fetal bovine serum (FBS) were purchased from Gibco-BRL Co. (Gaithersburg, MD, USA). ALA (at a purity of >99.5%) was purchased from Fushilai Medicine & Chemical Co., Ltd. (Changshu, China). The DCFH-DA fluorescent probe was purchased from Beyotime Institute of Biotechnology (Haimen, China). Protein

extract assay kits were obtained from KeyGEN Biotechnology (Nanjing, China). Antibodies specific for Bcl-2, caspase-3, ER α , ER β , ICAM-1, VCAM-1, I κ B α , phosphorylated p65 (p-p65), and Nox4 were obtained from Proteintech Group (Wuhan, China), and antibodies specific for p65, vinculin were purchased from Bioworld Technology (Nanjing, China). The NF- κ B inhibitor PDTC was obtained from Beyotime (Jiangsu, China), and the Nox4 inhibitor DPI and estrogen receptor inhibitor ICI182, 780 were purchased from Sigma (St. Louis, MO, USA).

Cell culture and H₂O₂ treatment

HAECs were cultured in DMEM containing 10% FBS at 37°C in 5% CO₂. After reaching 60% confluence, HAECs were treated with different concentrations of ALA (75, 150, 300 μ M) for 24 h before treatment with H₂O₂ (1 mM) for 4 h. ALA was dissolved in dimethylsulfoxide.

Western blotting analysis

HAECs or tissues were collected for protein extraction and detection of Bcl-2, caspase-3, ER α , ER β , ICAM-1, VCAM-1, I κ B α , p-p65, Nox4, total p65, and vinculin using a protein extraction kit (KeyGEN Biotechnology). HAECs were treated with different concentrations of ALA (75, 150, 300 μ M) for 24 h before treatment with H₂O₂ (1 mM) for 4 h. Mice were fasted overnight and sacrificed, aortas were dissected and separated from fatty tissue, and aortic protein was extracted using a protein extraction kit containing protease inhibitor and protein phosphatase inhibitor cocktail. Proteins (20–30 μ g per lane) were loaded on an 8–12% sodium dodecyl sulfate polyacrylamide electrophoresis gel, electrophoresed at 100 V for 2 h in gel running buffer for separation, and subsequently transferred to polyvinylidene fluoride (PVDF) membranes. Thereafter, membranes were blocked using 5% fat-free milk dissolved in Tris-buffered saline with Tween-20 (TBS-T) for 2 h at room temperature and then incubated at 4°C overnight with primary antibodies against Bcl-2 (Proteintech Group, Wuhan, China, 1:750 dilution), caspase-3 (Proteintech Group, 1:750 dilution), cleaved caspase-3 (Proteintech Group, 1:750 dilution), ER α (Proteintech Group, 1:500 dilution), ER β (Proteintech Group, 1:1000 dilution), ICAM-1 (Proteintech Group, 1:500 dilution), VCAM-1 (Proteintech Group, 1:500 dilution), I κ B α (Proteintech Group, 1:750 dilution), p-p65 (Proteintech Group, 1:750 dilution), Nox4 (Proteintech Group, 1:750 dilution and Affinity Biosciences, 1:750 dilution), total p65 (Bioworld Technology, 1:1000 dilution), and vinculin (Bioworld Technology, 1:5000 dilution). After a series of rinses, PVDF membranes were incubated with a peroxidase-conjugated secondary antibody (Bioworld Technology, 1:5000 dilution) in TBS-T for 2 h at room temperature. Finally, proteins were visualized using a chemiluminescence method (Gel DocTMXR, Bio-Rad, Hercules, CA, USA) and the protein levels were evaluated semiquantitatively using Image Lab software.

Immunofluorescence staining

At indicated times of differentiation, cultured cells were washed with phosphate buffered saline (PBS) twice and fixed with 4% paraformaldehyde/PBS for 10 min at room temperature. Then, cells were permeabilized with 0.5% Triton-X/PBS for 10 min. After washing with PBS, cells were blocked with 2% bovine serum albumin and incubated overnight at 4°C with primary antibodies, including ER α (Proteintech Group, 1:50 dilution), and p65 (Bioworld Technology, 1:50 dilution). Secondary antibodies including Alexa Fluor 488-conjugated anti-rabbit IgG (goat, 1:100, Thermo Fisher Scientific, Waltham, MA, USA) were used according to the manufacturer's instructions. The samples were incubated at room temperature for 1 h. After washing with PBS and counterstaining with DAPI, samples were mounted with Prolong Gold antifade solution (Thermo Fisher Scientific). Cells were visualized and photomicrographs were obtained using an upright fluorescence microscope (Olympus, Center Valley, PA, USA). Quantification of fluorescence intensity was performed using ImageJ. At least 50 cells from three different areas per chamber were measured.

Biochemical analysis of HAECs and serum

We used a colorimetric assay kit (Beyotime, Nanjing, China) to measure lactic dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), and L-glutathione (GSH) release. HAECs were cultured in 6-well plates at a density of 1 $\times$ 10⁵ per well and treated with different concentrations of ALA (75, 150, 300 μ M) for 24 h before stimulating with H₂O₂ (1mM) for 4 h.

Biomarkers of oxidative stress and antioxidants in serum, including LDH, MDA, SOD, and GSH, were detected using the abovementioned assay kits. Biomarkers of lipid metabolism in serum, including total

cholesterol (CHO), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), were measured using assay kits (Beyotime, Nanjing, China).

Monocyte adhesion assay

THP-1 cells (a human acute monocytic leukemia cell line) were labeled with the fluorescent probe BCECF-AM (Beyotime, Jiangsu, China) then incubated with confluent HAECs for 1 h at 37°C. Non-adherent THP-1 cells were removed gently with PBS. Images of adherent THP-1 cell were obtained using fluorescence microscopy (Olympus; x100 magnification).

Measurement of intracellular ROS

The level of ROS was detected using the fluorescent probe, 2, 7-dichlorofluorescein diacetate (DCFH-DA). A final concentration of 10 μM DCFH-DA was added to the HAECs, and cells were washed twice with serum-free medium, and then incubated for 20 min at 37°C in the dark. The relative levels of fluorescence were quantified using a FACScalibur Flow Cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

TUNEL staining

Cell apoptosis in plaques was examined using TUNEL ApopTag Fluorescein in Situ Apoptosis Detection Kit (Biotool, Houston, TX, USA). Freshly cut cryosections were fixed with 1% paraformaldehyde in PBS for 10 min at room temperature, rinsed with PBS, and permeabilized with a mixture of ethanol and acetic acid (2:1) for 5 min at -20°C. Slides were incubated with reaction buffer containing the TdT enzyme for 1 h at 37°C in a humidified chamber and then incubated with a FITC-conjugated anti-digoxigenin antibody for 30 min at room temperature. Finally, slides were mounted in a DAPI/antifade-containing medium. Slides were observed and photographed with an Olympus BX61 fluorescence microscope.

Histology and immunohistochemistry

Successive sections were collected on the same slide and at least 15 slides from 5 consecutive mouse thoracic aortas per area per mouse were examined. Aortas were dissected and separated from fatty tissue. After Oil-Red-O staining, we used Image-Pro Plus analysis to measure the proportion of lipid deposition. Mouse aortas were fixed in 10% formalin, embedded in paraffin, and sectioned (5 μm thickness). Sections were treated with xylene to remove the paraffin and were rehydrated, incubated with 3% H₂O₂ for 10 min, and then washed three times with PBS. Next, the sections were blocked with serum for 30 min and incubated with primary antibodies overnight against Nox4 (Proteintech Group, 1:100 dilution), p-p65 (Proteintech Group, 1:100 dilution), ICAM-1 (Proteintech Group, 1:50 dilution), VCAM-1 (Proteintech Group, 1:50 dilution), and CD68 (Abcam, Cambridge, MA, USA 1:200 dilution). Images were captured using Leica Microsystems.

Elastin staining

Elastin in the murine arterial wall was stained using Gomori's aldehyde-fuchsin staining method, with an elastic fiber staining kit (Maixin Bio, Fuzhou, China). Briefly, after deparaffinization and rehydration, sections were incubated for 5 min in Lugol's iodine solution, washed with PBS and incubated with sodium thiosulphate for 5 min. After washing with PBS and 70% ethanol, sections were incubated with aldehyde-fuchsin for 10 min and acid Orange G for seconds.

Animals and experimental design

Forty 16-week-old *Ldlr*^{-/-} female mice with a C57BL/6 background were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All experimental procedures were performed in accordance with the Institutional Animal Care and Use Committee of the Center for Laboratory Animal Sciences, Dalian Medical University, China. The mice were randomly divided into five groups: a sham surgery group (SHAM); a SHAM+high fat diet (HFD) group, and three ovariectomized (OVX) groups; the OVX mice were then randomly divided into three groups of eight animals per group: (1) OVX+HFD group; (2) OVX+ALA 70 mg/kg; (3) OVX+ALA 210 mg/kg. Mice in the three OVX groups underwent ovariectomy during the first week of the experiment, and 1 week later, the two OVX+ALA groups were treated with ALA (dissolved in 0.5% carboxymethylcellulose [CMC-Na], 3.75 ml/kg body weight per day) by gavage once daily. Mice in the

SHAM and SHAM+HFD group were treated with vehicle (0.5% CMC-Na, 3.75 ml/kg body weight) by gavage once daily. All mice except those in the SHAM group were fed an HFD. The feeding regimens and ALA gavage continued for 16 weeks. The body weight of each mouse was monitored weekly. After 16 weeks, the mice were fasted overnight and sacrificed, and blood samples were obtained from the retro-orbital plexus, and centrifuged at 1200 g for 10 min at 4°C. Aortas were dissected and separated from fatty tissue. After Oil-Red-O staining, we used Image-Pro Plus to measure the proportion of lipid deposition. Subsequently, the serum was frozen at -80°C for later use.

Statistical analysis

Results are expressed as the mean $\pm$ SEM or SD. The *in vitro* assay was performed at least three times. Statistical analysis was performed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). Data were compared between groups using a Student t-test or one-way ANOVA analysis of variance, followed by the least significant difference post-test. Statistical significance was set at $P < 0.05$.

Results

ALA prevented OVX- and/or HFD- induced atherosclerosis

The *Ldlr*^{-/-} mouse is a widely used animal model of atherosclerosis because it demonstrates a sensitive response to oxidative stress and inflammation and naturally develops atherosclerosis [36]. To simulate the development of atherosclerotic lesions in postmenopausal women, we performed OVX in 16-week-old *Ldlr*^{-/-} female mice and subsequently fed them a HFD. To evaluate the effects of ALA treatment on the development of atherosclerosis in the OVX *Ldlr*^{-/-} mouse model and/or induced by a HFD, these mice received ALA by oral gavage from the second week after OVX. We observed more body weight gain in the OVX *Ldlr*^{-/-} group fed a HFD than in the control SHAM+HFD group without OVX; weight gain in both of these groups was substantially higher than in the SHAM group fed normal chow without OVX (Fig. 1A). However, ALA treatment markedly reduced the body weight of the OVX *Ldlr*^{-/-} group on a HFD, especially after a prolonged period of ALA administration (Fig. 1A). The *Ldlr*^{-/-} mice that underwent OVX and received a HFD developed markedly more

Fig. 1. ALA reversed OVX and HFD induced atherosclerosis. At the end of 16 week treatment, mice were fasted overnight and sacrificed, aortas were dissected and separated from fatty tissue. (A) Effect of ALA on the body weight of mice. $^{**}P < 0.01$, OVX+HFD+ALA(L) group compared to the OVX+HFD group; $^{***}P < 0.001$, OVX+HFD+ALA(H) group compared to the OVX+HFD group. (B) Gross anatomy of aorta and oil-red-O staining. (C) Scatter plot of the aortic atherosclerotic plaque was quantified using an image-pro plus analysis system. $^{**}P < 0.01$, SHAM+HFD compared to the SHAM group, OVX+HFD+ALA(L) group compared to the OVX+HFD group; $^{***}P < 0.001$, OVX+HFD+ALA(H) group compared to the OVX+HFD group. (D) Serial paraffin sections of aortas were stained with hematoxylin-eosin (HE), Elastica van Gieson (EVG). Scale bars=100 μ m. Data were expressed as the mean $\pm$ SEM (n=8, each group).

severe atherosclerotic lesions with fatty streaks and dots covering the thoracic aorta and aortic arch/sinus than did the *Ldlr*^{-/-} SHAM+HFD group (Fig. 1B). Microscopically, the ALA treated group exhibited drastic reductions in plaque size and lipid deposition (Fig. 1C). In addition, histological sections showed that ALA treatment significantly attenuated arterial wall thickness, protecting the internal elastic fibers from fragmenting and disrupting (Fig. 1D). Taken together, these results demonstrate that ALA prevented the development of atherosclerosis induced by OVX and HFD.

ALA reversed hyperlipidemia and oxidative stress induced by OVX and HFD

Next, we evaluated the effects of ALA on elevated serum lipid levels, one of the risk factors for atherosclerosis. Mice in the OVX+HFD group had higher serum TG, CHO, and LDL-C levels but lower levels of HDL-C compared with the SHAM+HFD group, suggesting that menopause played a role in hyperlipidemia. However, the administration of ALA significantly decreased the levels of TG, CHO, and LDL-C while elevating the level of HDL-C (Fig. 2A-D). These results demonstrate that the hyperlipidemia induced by HFD and OVX was prevented by ALA.

Accumulating evidence suggests that oxidative stress plays an important role in the process of atherosclerosis. Therefore, we measured several biomarkers of oxidative damage and antioxidants, including LDH, a representative marker of oxidative injury, MDA, a marker of lipid-peroxidation [37], and antioxidant enzymes SOD and GSH [38] in the *Ldlr*^{-/-} animal model. We found that OVX and HFD suppressed the levels of SOD and GSH activity while elevating the levels of LDH and MDA in *Ldlr*^{-/-} mice, suggesting menopause and an HFD contributed to oxidative stress. Of note, ALA treatment significantly decreased the levels of biomarkers of oxidative stress but enhanced the levels of antioxidant enzymes in mouse serum (Fig. 2E-H). Taken together, our data indicate that ALA administration prevented the development of atherosclerosis by inhibiting hyperlipidemia and oxidative stress in HFD/OVX *Ldlr*^{-/-} mice.

ALA up-regulated the expression of estrogen receptors in OVX mice

ERs can activate several cellular kinases by inducing a “rapid” non-nuclear signaling cascade independent of estrogen [36]. We found that protein levels of ER α and ER β were markedly enhanced after treatment with ALA in a dose- and time-dependent manner (Fig. 3A-C). We next confirmed this observation with an immunofluorescent assay, in which ALA reversed the down-regulation of ER α expression induced by H₂O₂ treatment (Fig. 3D). To further validate our findings, we examined the expression of ER in *Ldlr*^{-/-} mice. We found that the expression of ER α and ER β decreased significantly in HFD/OVX *Ldlr*^{-/-} mice. However, ALA

Fig. 2. Effects of ALA on the serum biochemical parameters of oxidative and anti-oxidative status as well as lipid metabolism in vivo. At the end of 16 week treatment, mice were fasted overnight and sacrificed, blood samples were obtained from the retro-orbital plexus. Plasma levels of TG (A); CHO (B); HDL-C (C); LDL-C (D); LDH (E); MDA (F); SOD (G); GSH (H) were measured by enzymatic methods using commercial test kit (Beyotime, Nanjing, China). All experiments were performed independently three times. #P<0.05 and ##P<0.01 compared to the SHAM group. ΔP<0.05 compared to the SHAM+HFD group. **P<0.01 compared to the OVX+HFD group. Data were expressed as the mean ± SEM (n=8, each group).

Fig. 3. Effects of ALA on the protein levels of estrogen receptor- α (ER α) and estrogen receptor- β (ER β). ALA up-regulated ER α and ER β expression both in dose-dependent (A)(B) and time-dependent (C) way which were detected by western blot and immunofluorescence (D) in HAECs. HAECs were stained for ER α which exhibited red fluorescence. Scale bars=20 μ m. The fluorescence intensity was measured by the ImageJ software. Results from one representative experiment (out of two) were shown. At the end of 16 week treatment, mice were fasted overnight and sacrificed, aortas were dissected and separated from fatty tissue, then aorta protein was extracted using a protein extraction kit containing protease inhibitor and protein phosphatase inhibitor cocktail. The expression of ER α (E) and ER β (F) in arteries of mice was measured by western blot. In vitro assay, $^{##}P<0.01$ compared to the group without H₂O₂ and ALA. $^{*}P<0.05$ and $^{**}P<0.01$ compared to the group treated with H₂O₂ alone. Data were expressed as the mean $\pm$ SD. All experiments were performed independently three times. In vivo assay, $^{##}P<0.01$ compared to the SHAM group. $^{*}P<0.05$ and $^{**}P<0.01$ compared to the OVX+HFD group. Data were expressed as the mean $\pm$ SEM (n=8, each group).

treatment significantly elevated the expression of estrogen receptors in a dose-dependent manner, which is consistent with our *in vitro* findings (Fig. 3E-F). These results suggest that ALA may up-regulate the expression of ER α and ER β that was diminished during the development of atherosclerosis.

ALA prevented H₂O₂-induced oxidative stress through down-regulating the expression of Nox4 and p22phox.

Oxidative stress is mainly derived from excessive generation of ROS, which plays a key role in the progression of atherosclerosis [2, 13, 14]. To evaluate the effects of ALA on eliminating intracellular ROS, we measured changes in ROS levels in response to ALA treatment by flow cytometry and fluorescence microscopy. We found that intracellular ROS levels were substantially reduced following ALA treatment, suggesting that ALA eliminated the excessive generation of intracellular ROS stimulated by H₂O₂ (Fig. 4A-B). We next examined the protein levels of Nox4 and p22phox, two key components in ROS-generation, and found that ALA treatment markedly abrogated Nox4 and p22phox expression induced by H₂O₂ (Fig. 4C-D). In the *Ldlr*^{-/-} mouse model, the expression of Nox4 in the SHAM+HFD and OVX+HFD groups was remarkably elevated compared to the SHAM group. However, ALA administration significantly decreased the expression of Nox4 in OVX+HFD *Ldlr*^{-/-} mice in a dose-dependent manner (Fig. 4E). Immunohistochemical staining showed similar results as the western blot analysis (Fig. 4F). These findings suggest that ALA might exert its anti-oxidative effects by suppressing the expression of ROS-generating enzymes such as Nox4 and p22phox.

ALA protected against H₂O₂-induced injury by inhibiting the NF- κ B pathway

The ROS-NF- κ B signaling pathway plays a crucial role in activating inflammatory and immune responses that contribute to the pathogenesis of atherosclerosis [17]. It has been

Fig. 4. ALA eliminated the excessive generation of intracellular ROS stimulated by H₂O₂ and down-regulated Nox4 and p22phox protein synthesis in vitro and in vivo. (A) (B) ALA eliminated excessive generation of intracellular ROS (exhibited as green fluorescence) which was labeled by DCFH-DA in HAECs. Scale bars=50μm. Results from one representative experiment (out of two) were shown. (C) (D) ALA down-regulated Nox4 and p22phox protein level in HAECs. At the end of 16 week treatment, mice were fasted overnight and sacrificed, aortas were dissected and separated from fatty tissue, then aorta protein was extracted using a protein extraction kit containing protease inhibitor and protein phosphatase inhibitor cocktail. The expression of Nox4 in

mice was evaluated by western blot (E) and immunohistochemistry (F). Scale bars=100μm. In vitro assay, [#]P<0.05 and ^{##}P<0.01 compared to the group without H₂O₂ and ALA. ^{*}P<0.05 and ^{**}P<0.01 compared to the group treated with H₂O₂ alone. Data were expressed as the mean ± SD. All experiments were performed independently three times. In vivo assay, [#]P<0.05 and ^{###}P<0.001 compared to the SHAM group. ^{*}P<0.05 and ^{**}P<0.001 compared to the OVX+HFD group. Data were expressed as the mean ± SEM (n=8, each group).

reported that IκB phosphorylation and its subsequent ubiquitin-mediated proteasomal degradation translocate NF-κB dimers to the nucleus, leading to NF-κB activation and induction of NF-κB- dependent transcription [39]. To investigate the effect of ALA on H₂O₂-induced NF-κB activation in HAECs, we examined the protein levels of IκBα and p-p65. We found that ALA markedly suppressed the phosphorylation of p65 and the degradation of cytoplasmic IκBα compared with the H₂O₂ treatment group (Fig. 5A-B). To further evaluate the effects of ALA on NF-κB activation, we examined the cellular localization of its transcriptionally active subunit p65 in an immunofluorescence assay. We found that exogenous H₂O₂ treatment markedly stimulated p65 translocation into the nucleus, while ALA treatment led to the removal of p65 out of the nucleus in a dose-dependent manner (Fig. 5C). Consistent with *in vitro* findings, p-p65 was markedly increased in the OVX group compared with the SHAM group, while ALA treatment markedly reversed this change (Fig. 5D-E). These results demonstrated that oxidative stress injury induced by H₂O₂ or HFD/OVX could activate the NF-κB signaling pathway that contributes to the pathogenesis of atherosclerosis, whereas ALA could effectively inhibit activation of this pathway, providing a protective effect against atherosclerosis.

Effects of ALA on H₂O₂-induced adhesion in association with suppression of ROS, ER and NF-κB

The adhesion molecules ICAM-1 and VCAM-1 that are secreted by activated endothelial cells play a key role in initiating atherosclerosis and development of fatty streak lesions [40]. To determine whether Nox4, ERs, and NF-κB modulate ICAM-1 and VCAM-1 to promote atherosclerosis, we measured the expression of ICAM-1 and VCAM-1 in HAECs induced by H₂O₂ in the presence of Nox4, ERs, and NF-κB inhibitors. We found the increased expression

Fig. 5. Inhibiting effects of ALA on H₂O₂-induced NF-κB activation in HAECs and in OVX&HFD mice. (A) (B) Effect of ALA on the cytoplasmic IκBα and phosphorylation of p65 level of HAECs. (C) Effect of ALA on p65 localization and expression in HAECs. HAECs were stained with a p65 antibody and DAPI to visualize nuclei, and analyzed by immunofluorescence microscopy. p65 exhibited as red fluorescence, DAPI exhibited as blue fluorescence. Scale bars=20μm. The fluorescence intensity was measured by the ImageJ software. Results from one representative experiment (out of two) were shown. The expression of phosphorylated p65 in mice was evaluated by western blot (D) and immunohistochemistry (E). Scale bars=100μm. In vitro assay, ##P<0.01 compared to the group without H₂O₂ and ALA. *P<0.05, **P<0.01 and ***P<0.001

compared to the group treated with H₂O₂ alone. Data were expressed as the mean ± SD. All experiments were performed independently three times. In vivo assay, ###P<0.001 compared to the SHAM group. ***P<0.001 compared to the OVX+HFD group. Data were expressed as the mean ± SEM (n=8, each group).

of ICAM-1 and VCAM-1 induced by H₂O₂ was abrogated by the Nox4 inhibitor DPI and the NF-κB inhibitor PDTC, respectively, but was intensified in response to treatment with the estrogen receptor antagonist ICI182, 780 (Fig. 6A-C). These findings suggest that Nox4 and NF-κB played a positive role in promoting atherosclerosis while ER functioned as a negative regulator in the pathogenesis of atherosclerosis. Next, we investigated the effects of ALA on ICAM-1 and VCAM-1 by treating H₂O₂-induced HAECs with ALA as a single agent or with each of ALA+DPI, ALA+ICI, and ALA+PDTC. As expected, we found that ALA alone or in combination with DPI, ICI, or PDTC significantly suppressed the expression levels of ICAM-1 and VCAM-1 in H₂O₂-induced HAECs (Fig. 6A-C), indicating that ALA had protective effects against atherosclerosis by inhibiting the expression of ICAM-1 and VCAM-1. These results suggest that estrogen receptors might play a role in preventing postmenopausal atherosclerosis. In contrast, Nox4 and NF-κB promoted atherosclerosis.

As shown above, ROS could activate the NF-κB signaling pathway while suppressing estrogen receptors expression. As a primary source of ROS, Nox4 is highly expressed in the endothelium and interacts with NF-κB and ERs to form a ternary complex in the regulation of H₂O₂-induced oxidative stress. We next explored the relationships among Nox4, NF-κB, and ERs in response to oxidative stress. We found that PDTC down-regulated H₂O₂-induced Nox4 overexpression while up-regulating ERα expression upon H₂O₂ stimulation (Fig. 6D-E). In addition, as expected, DPI markedly reduced p-p65 levels and promoted ERα expression upon H₂O₂-induction (Fig. 6F-G). However, ICI182, 780 treatment significantly increased the levels of Nox4 and p-p65 in response to H₂O₂ stimulation (Fig. 6H-I), indicating that inhibition of ERs activated the NF-κB pathway and stimulated Nox4 overexpression. Immunofluorescence staining showed that ICI182, 780 treatment resulted in the translocation of p65 into the nucleus in HAECs upon H₂O₂ induction, but that ALA treatment blocked this process, with

Fig. 6. Effects of ALA paired with inhibitors of ROS, ERs and NF- κ B on adhesion molecules. DPI (10 μ M) or ICI182, 780 (100nM) or PDTC (50 μ M) was added in ALA-treated HAECs supernatant for 2h after being stimulated with H₂O₂ (1.0mM) for 2h to see the effect of ALA allied with DPI (A) or ICI182, 780 (B) or PDTC (C) on the expression of ICAM-1 and VCAM-1. The protein level of Nox4 (D) and ER α (E) treated with PDTC and/or H₂O₂. The expression of phosphorylated p65 (F) and ER α (G) reacted with DPI and/or H₂O₂. The protein level of Nox4 (H) and phosphorylated p65 (I) treated with ICI182, 780 and/or H₂O₂. (J) Effect of ALA and/or ICI182, 780 on p65 localization and expression in HAECs. HAECs were stained with a p65 antibody and DAPI to visualize nuclei, and analyzed by immunofluorescence microscopy. p65 exhibited as red fluorescence, DAPI exhibited as blue fluorescence. Scale bars=20 μ m. Results from one representative experiment (out of two) were shown. All experiments were performed independently three times. ##P<0.01 compared to the group treated with H₂O₂ alone. *P<0.05 and **P<0.01 compared to the group treated with H₂O₂ and inhibitors. Data were expressed as the mean $\pm$ SD.

p65 remaining in the cytoplasm (Fig. 6J). Based on our findings, we propose that Nox4, NF- κ B, and ERs might interact to form a ternary complex with ROS to modulate the atherosclerotic process.

ALA protected arterial endothelial cells from apoptosis.

It has been reported that endothelial injury and apoptosis lead to lipid accumulation and inflammation which contribute to atherosclerotic plaque formation [41]. In postmenopausal women, estrogen deficiency promotes caspase-3 dependent apoptosis [42]. To investigate the effects of ALA on endothelial apoptosis, we measured the protein levels of cleaved caspase-3 and Bcl-2 under oxidative stress. We found that ALA treatment significantly reduced the expression levels of cleaved caspase-3 and enhanced Bcl-2 expression in HAECs upon H₂O₂ induction (Fig. 7A-B). In an vivo study, we showed that OVX Ldlr^{-/-} mice receiving HFD developed atherosclerosis with a significant increase in cellular apoptosis as evidence by TUNEL staining. Notably, ALA treatment markedly inhibited apoptosis in the arterial wall of HFD/OVX Ldlr^{-/-} mice in a dose dependent manner (Fig. 7C). Together, these findings indicate that ALA exerted its anti-apoptotic functions by up-regulating prosurvival Bcl-2 protein levels and down-regulating pro-apoptotic cleaved caspase-3 levels in arterial endothelial cells.

Cell adhesion molecules such as ICAM-1 and VCAM-1 play an important role in initiation and development of fatty streak lesions in the arterial wall [43]. As shown in Fig. 7D-E, H₂O₂ stimulation significantly increased the expression of ICAM-1 and VCAM-1 in HAECs. However, ALA treatment markedly and dose-dependently attenuated the expression of ICAM-1 and VCAM-1 (Fig. 7D-E). In the monocyte adhesion assay, H₂O₂ induction resulted in a striking increase in THP-1 adhesion to HAECs (Fig. 7F), while ALA treatment markedly suppressed this effect in a dose-dependent manner. Consistently, immunohistochemical staining showed that OVX+HFD Ldlr^{-/-} mice exhibited significantly higher expression of ICAM-1 and VCAM-1 than the SHAM or SHAM+HFD groups, whereas ALA administration substantially reduced

Fig. 7. ALA defended cells and tissues from undergoing apoptosis and blocked monocyte adhesion to endothelial cells through inhibiting ICAM-1 and VCAM-1 expressions. Cells were pretreated with different concentrations of ALA (75, 150, 300 μ M) for 24h before being stimulated with H_2O_2 (1.0mM) for 4h, and the protein expression of Cleaved Caspase-3 (A) and Bcl-2 (B) were analyzed. (C) Arteries undergoing apoptosis were positively stained with TUNEL reagent and are shown in green. The group of OVX+HFD showed the most obvious apoptosis, which could be reversed by ALA. Scale bars=100 μ m. The fluorescence intensity was measured by the ImageJ software. Results from one representative experiment (out of two) were shown. The protein expression of ICAM-1 (D) and VCAM-1 (E) were determined. ICAM-1 and VCAM-1 were down-regulated by ALA treatment. (F) Most majority of THP-1 cells labeled with BCECF (exhibited as green fluorescence) adhered to the group treated with H_2O_2 alone, which could be reversed gradually by ALA in an increasing concentration. Scale bars=50 μ m. The fluorescence intensity was measured by the ImageJ software. Results from one representative experiment (out of two) were shown. (G) Immunohistochemistry staining of ICAM-1, VCAM-1 and CD68 (H). Scale bars=100 μ m. In vitro assay, [#]*P*<0.01 compared to the group without H_2O_2 and ALA. **P*<0.05, ***P*<0.01 and ****P*<0.001 compared to the group treated with H_2O_2 alone. Data were expressed as the mean $\pm$ SD. All experiments were performed independently three times. In vivo assay, [#]*P*<0.01 and ^{###}*P*<0.001 compared to the SHAM group. ****P*<0.001 compared to the OVX+HFD group. Data were expressed as the mean $\pm$ SEM (n=8, each group).

the expression of ICAM-1 and VCAM-1 in OVX+HFD *Ldlr*^{-/-} mice in a dose-dependent manner (Fig. 7G). Accumulating evidence suggests that macrophages play an essential role in the formation and development of atherosclerotic plaques [44]. Here we used CD68 staining, a representative marker of activation of the mononuclear-phagocyte system, to show the presence of macrophages in our *in vivo* atherosclerotic model. We found that ALA treatment significantly inhibited atherosclerosis-associated macrophages in the area of atherosclerotic plaques of OVX+HFD *Ldlr*^{-/-} mice in a dose-dependent manner (Fig. 7H). Collectively, these data suggest that ALA can block the activation of monocyte adhesion through suppressing

Fig. 8. ALA protected HAECs against oxidative injury in the absence of H₂O₂. HAECs were cultured in 6-well plates at a density of 1X10⁵ per well and treated with different concentrations of ALA (75, 150, 300μM) for 24 hours before stimulating with H₂O₂ (1mM) for 4 hours. We used a colorimetric assay kit to measure LDH, MDA, SOD, and GSH release. ALA down-regulated LDH activity (A), MDA level (B), but up-regulated SOD activity (C) and GSH level in HAECs. All experiments were performed independently three times. ##P<0.01 compared to the group without H₂O₂ and ALA. *P<0.05 and **P<0.01 compared to the group treated with H₂O₂ alone. Data were expressed as the mean ± SD.

ICAM-1 and VCAM-1 expression and the mononuclear-phagocyte system to prevent the pathogenesis of atherosclerosis.

ALA protected HAECs against oxidative injury in the presence of H₂O₂

LDH is a stable enzyme normally present in the cytosol of most eukaryotic cells but rapidly releases into the supernatant upon cell death due to damage of the plasma membrane [45]. We found that ALA treatment significantly inhibited LDH activity of HAECs induced by H₂O₂ stimulation (Fig. 8A). To further evaluate the antioxidative effects of ALA, we measured the levels of MDA, SOD and GSH in HAECs in response to H₂O₂ stimulation. We showed that ALA treatment significantly suppressed MDA levels while elevating SOD activity and GSH levels in HAECs during oxidative injury (Fig. 8B-D). Taken together, our findings suggest that ALA provided antioxidant protection and prevention against oxidative damage-induced endothelial cell death.

Discussion

Atherosclerosis is a complex pathological process modulated by multiple factors such as inflammation, oxidative stress, and apoptosis [46-48]. It has become one of the most common chronic vascular diseases worldwide and is an important public health concern in modern society, especially for postmenopausal women. It is well known that postmenopausal women exhibit a higher incidence of cardiovascular disease than premenopausal women due to loss of responsiveness to either internal or even externally provided estrogen [3]. HRT in postmenopausal women for the purpose of primary or secondary prevention of cardiovascular diseases is not effective, and is associated with an increase in the risk of developing stroke and venous thromboembolic events [49]. Estrogen may have a beneficial effect if initiated early during menopause, when a woman's arteries are likely to be relatively healthy, but it has a deleterious effect if started in late menopause, when the arteries are more likely to exhibit signs of atherosclerotic disease [50]. ALA, a natural antioxidant synthesized in the mitochondria of liver and other tissues [51], has multiple anti-inflammatory and anti-oxidative effects. In this study, we found that ALA stimulated dose- and time-dependent expression of estrogen receptors. Consequently, ALA may be used as a naturally occurring free radical scavenger to replace HRT for postmenopausal women.

It is well established that the NF-κB signaling pathway has a role in a number of physiological process, involving immune inflammatory responses, oxidative stress, cell adhesion, differentiation, and apoptosis [52-54], many of which contribute to atherosclerosis. NF-κB can be activated by oxidative stress and inflammation, but on the other hand, activation of NF-κB up-regulates the genes modulating oxidative stress, inflammation, cell proliferation, and apoptosis, providing a feedback control [55]. Therefore, activation of NF-

κB plays an essential role in the pathogenesis of atherosclerosis. In this study, we showed that ALA treatment inhibited NF-κB activation upon H₂O₂ induction *in vitro* and OVX/HFD *in vivo* by suppressing phosphorylation of p65 and the degradation of cytoplasmic IκBα, suggesting that ALA prevents atherosclerosis, at least in part, through inhibiting the NF-κB signaling pathway.

ROS is the main product of oxidative stress, which has a role in multiple pathological process in the development of cardiovascular diseases [56]. It was reported that estrogen deficiency led to an increase in ROS generation, implying that postmenopausal women may have a greater risk of oxidative stress [7]. Many cardiovascular diseases are known to be associated with elevated ROS levels. A major source of cellular ROS is the Nox family of enzymes, comprising seven members [57]. Nox4 is the major isoform that is constitutively active and expressed in endothelial cells and vascular smooth muscle cells [58]. Nox4-derived ROS production is considered to be regulated mainly through alterations in Nox4 expression levels [59]. Nox4 colocalizes with the integral membrane protein p22phox which is necessary for Nox4 activity [60]. In this study, we showed that ALA treatment significantly reduced the over-expression of Nox4 and p22phox in response to H₂O₂ stimulation. Moreover, we demonstrated that ALA diminished the excessive generation of intracellular ROS induced by H₂O₂. Therefore, ALA may modulate the ROS-generating enzymes to exert its potent anti-oxidative effects and provide protection against atherosclerosis.

Endothelial cell apoptosis plays an important role in the formation and progression of atherosclerotic lesions [61]. Estrogen deficiency has been reported to facilitate caspase-3-dependent apoptosis in postmenopausal women [8]. In the present study, we demonstrated that ALA treatment was sufficient to reverse H₂O₂-induced apoptosis and cellular injury in HAECs, and OVX/HFD induced apoptosis in atherosclerotic lesions of *Ldlr*^{-/-} mice. As such, one of the molecular mechanisms responsible for the atheroprotective effects of ALA likely involves repression of cellular apoptotic pathway during the pathogenesis of atherosclerosis.

ICAM-1 and VCAM-1 are endothelial adhesion molecules that play a key role in monocyte accumulation/adhesion to the endothelium and are important at all stages of atherogenesis. Here, we clearly showed that ALA suppressed the expression of ICAM-1 and VCAM-1 both *in vivo* and *in vitro*, implying that ALA could disturb one of the key elements in the pathophysiology of atherosclerotic disease, monocyte adhesion, through inhibiting ICAM-1 and VCAM-1 expression.

A significant body of evidence has shown that postmenopausal women have a higher incidence of atherosclerosis than premenopausal women [3]. Among multiple speculations and hypotheses, estradiol is considered to play a central role [3]. In the present study, we showed that ALA treatment up-regulated the protein levels of estrogen receptors both *in vivo* and *in vitro*. We also showed that H₂O₂ stimulation down-regulated both ERα and ERβ expression in HAECs. Meanwhile, ERs expressions were decreased in atherosclerotic pathological tissue induced by HFD and/or OVX. Our results are in line with previous work showing that ERs expressions are modulated by oxidative stress and inflammation in cardiovascular disease [62]. Importantly, we found that ALA treatment markedly up-regulated estrogen receptor protein levels both *in vivo* and *in vitro*, suggesting that ALA exerts atheroprotective effects, at least partly, by promoting the expression of estrogen receptors.

It has previously been reported that NF-κB and Nox4 together up-regulate indoxyl sulfate-induced angiotensinogen expression in proximal tubular cells [63]. In the present study, Nox4 overexpression induced by H₂O₂ was down-regulated by the NF-κB inhibitor PDTC, suggesting that NF-κB activation elevated the expression of Nox4. Alterations of p-p65 induced by H₂O₂ were reversed by the Nox4 inhibitor DPI, indicating that Nox4 promoted NF-κB activation. The decreased expression of ERα and ERβ induced by H₂O₂ was restored by PDTC and DPI, suggesting that Nox4 and NF-κB inhibited the expression of ERs. Taken together, Nox4 and NF-κB likely cooperate to inhibit the expression of estrogen receptors, and jointly promote the development of atherosclerosis. ALA can abolish such an inhibitory effect by up-regulating the expression of ERs, consequently activating several cellular kinases by inducing a “rapid” non-nuclear signaling cascade independent of estrogen.

In summary, our findings suggest that estrogen receptors “communicate” with NF- κ B and Nox4 in the form of mutual restraint to participate in the regulation of initiation and development of atherosclerosis. ALA, a potent natural antioxidant which can enhance the expression of estrogen receptors and inhibit the activation of NF- κ B signaling, may be harnessed as a potential therapeutic candidate to treat atherosclerosis in postmenopausal women.

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Disclosure Statement

The authors declare to have no competing financial or commercial interests that could potentially lead to a conflict of interests.

References

- 1 Lusis AJ: Atherosclerosis. *Nature* 2000;407:233-241.
- 2 Libby P, Ridker PM, Maseri A: Inflammation and atherosclerosis. *Circulation* 2002;105:1135-1143.
- 3 Amant C, Holm P, Xu Sh SH, Tritman N, Kearney M, Losordo DW: Estrogen receptor-mediated, nitric oxide-dependent modulation of the immunologic barrier function of the endothelium: regulation of fas ligand expression by estradiol. *Circulation* 2001;104:2576-2581.
- 4 Liu X, Guo CY, Ma XJ, Wu CF, Zhang Y, Sun MY, Pan YT, Yin HJ: Anti-inflammatory effects of tanshinone IIA on atherosclerotic vessels of ovariectomized ApoE mice are mediated by estrogen receptor activation and through the ERK signaling pathway. *Cell Physiol Biochem* 2015;35:1744-1755.
- 5 Pelekanou V, Kampa M, Kiagiadaki F, Deli A, Theodoropoulos P, Agrogiannis G, Patsouris E, Tsapis A, Castanas E, Notas G: Estrogen anti-inflammatory activity on human monocytes is mediated through cross-talk between estrogen receptor ERalpha36 and GPR30/GPER1. *J Leukoc Biol* 2016;99:333-347.
- 6 Nakamura Y, Suzuki T, Miki Y, Tazawa C, Senzaki K, Moriya T, Saito H, Ishibashi T, Takahashi S, Yamada S, Sasano H: Estrogen receptors in atherosclerotic human aorta: inhibition of human vascular smooth muscle cell proliferation by estrogens. *Mol Cell Endocrinol* 2004;219:17-26.
- 7 Wing LY, Chen YC, Shih YY, Cheng JC, Lin YJ, Jiang MJ: Effects of oral estrogen on aortic ROS-generating and -scavenging enzymes and atherosclerosis in apoE-deficient mice. *Exp Biol Med* 2009;234:1037-1046.
- 8 Yu J, Eto M, Akishita M, Okabe T, Ouchi Y: A selective estrogen receptor modulator inhibits TNF-alpha-induced apoptosis by activating ERK1/2 signaling pathway in vascular endothelial cells. *Vascul Pharmacol* 2009;51:21-28.
- 9 Hodis HN, Mack WJ, Henderson VW, Shoupe D, Budoff MJ, Hwang-Levine J, Li Y, Feng M, Dustin L, Kono N, Stanczyk FZ, Selzer RH, Azen SP, Group ER: Vascular Effects of Early versus Late Postmenopausal Treatment with Estradiol. *N Engl J Med* 2016;374:1221-1231.
- 10 Keaney JF, Jr., Solomon CG: Postmenopausal Hormone Therapy and Atherosclerosis--Time Is of the Essence. *N Engl J Med* 2016;374:1279-1280.
- 11 Kassi E, Spilioti E, Nasiri-Ansari N, Adamopoulos C, Moutsatsou P, Papapanagiotou A, Siasos G, Tousoulis D, Papavassiliou AG: Vascular Inflammation and Atherosclerosis: The Role of Estrogen Receptors. *Curr Med Chem* 2015;22:2651-65.
- 12 Hodgins JB, Kregel JH, Reddick RL, Korach KS, Smithies O, Maeda N: Estrogen receptor alpha is a major mediator of 17beta-estradiol's atheroprotective effects on lesion size in Apoe^{-/-} mice. *J Clin Invest* 2001;107:333-340.

- 13 Gray SP, Di Marco E, Okabe J, Szyndralewicz C, Heitz F, Montezano AC, de Haan JB, Koulis C, El-Osta A, Andrews KL, Chin-Dusting JP, Touyz RM, Wingler K, Cooper ME, Schmidt HH, Jandeleit-Dahm KA: NADPH oxidase 1 plays a key role in diabetes mellitus-accelerated atherosclerosis. *Circulation* 2013;127:1888-1902.
- 14 Haendeler J, Eckers A, Lukosz M, Unfried K, Altschmied J: Endothelial NADPH oxidase 2: when does it matter in atherosclerosis? *Cardiovasc Res* 2012;94:1-2.
- 15 Zhen J, Lu H, Wang XQ, Vaziri ND, Zhou XJ: Upregulation of endothelial and inducible nitric oxide synthase expression by reactive oxygen species. *Am J Hypertens* 2008;21:28-34.
- 16 Lassegue B, Griendling KK: NADPH oxidases: functions and pathologies in the vasculature. *Arterioscler Thromb Vasc Biol* 2010;30:653-661.
- 17 Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, Packer L: Redox regulation of NF-kappa B activation. *Free Radic Biol Med* 1997;22:1115-1126.
- 18 Hayden MS, Ghosh S: Shared principles in NF-kappaB signaling. *Cell* 2008;132:344-362.
- 19 Shiraki A, Oyama J, Komoda H, Asaka M, Komatsu A, Sakuma M, Kodama K, Sakamoto Y, Kotooka N, Hirase T, Node K: The glucagon-like peptide 1 analog liraglutide reduces TNF-alpha-induced oxidative stress and inflammation in endothelial cells. *Atherosclerosis* 2012;221:375-382.
- 20 Manea A, Tanase LI, Raicu M, Simionescu M: Transcriptional regulation of NADPH oxidase isoforms, Nox1 and Nox4, by nuclear factor-kappaB in human aortic smooth muscle cells. *Biochem Biophys Res Commun* 2010;396:901-907.
- 21 Sudoh N, Toba K, Akishita M, Ako J, Hashimoto M, Iijima K, Kim S, Liang YQ, Ohike Y, Watanabe T, Yamazaki I, Yoshizumi M, Eto M, Ouchi Y: Estrogen prevents oxidative stress-induced endothelial cell apoptosis in rats. *Circulation* 2001;103:724-729.
- 22 Karas RH, Hodgin JB, Kwoun M, Krege JH, Aronovitz M, Mackey W, Gustafsson JA, Korach KS, Smithies O, Mendelsohn ME: Estrogen inhibits the vascular injury response in estrogen receptor beta-deficient female mice. *Proc Natl Acad Sci U S A* 1999;96:15133-15136.
- 23 O'Malley BW, Tsai MJ: Molecular pathways of steroid receptor action. *Biol Reprod* 1992;46:163-167.
- 24 Dantas AP, Tostes RC, Fortes ZB, Costa SG, Nigro D, Carvalho MH: *In vivo* evidence for antioxidant potential of estrogen in microvessels of female spontaneously hypertensive rats. *Hypertension* 2002;39:405-411.
- 25 Min J, Weitian Z, Peng C, Yan P, Bo Z, Yan W, Yun B, Xukai W: Correlation between insulin-induced estrogen receptor methylation and atherosclerosis. *Cardiovasc Diabetol* 2016;15:156.
- 26 Nevzati E, Shafighi M, Bakhtian KD, Treiber H, Fandino J, Fathi AR: Estrogen induces nitric oxide production via nitric oxide synthase activation in endothelial cells. *Acta Neurochir Suppl* 2015;120:141-145.
- 27 Li QY, Chen L, Zhu YH, Zhang M, Wang YP, Wang MW: Involvement of estrogen receptor-beta in farnesol inhibition of rat thoracic aorta vascular smooth muscle cell proliferation. *Acta Pharmacol Sin* 2011;32:433-440.
- 28 Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J, Writing Group for the Women's Health Initiative I: Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *Jama* 2002;288:321-333.
- 29 Santen RJ, Allred DC, Ardoin SP, Archer DF, Boyd N, Braunstein GD, Burger HG, Colditz GA, Davis SR, Gambacciani M, Gower BA, Henderson VW, Jarjour WN, Karas RH, Kleerekoper M, Lobo RA, Manson JE, Marsden J, Martin KA, Martin L, Pinkerton JV, Rubinow DR, Teede H, Thiboutot DM, Utian WH, Endocrine S: Postmenopausal hormone therapy: an Endocrine Society scientific statement. *J Clin Endocrinol Metab* 2010;95:s1-s66.
- 30 Jones W, Li X, Qu ZC, Perriott L, Whitesell RR, May JM: Uptake, recycling, and antioxidant actions of alpha-lipoic acid in endothelial cells. *Free Radic Biol Med* 2002;33:83-93.
- 31 Lv C, Maharjan S, Wang Q, Sun Y, Han X, Wang S, Mao Z, Xin Y, Zhang B: alpha-Lipoic Acid Promotes Neurological Recovery After Ischemic Stroke by Activating the Nrf2/HO-1 Pathway to Attenuate Oxidative Damage. *Cell Physiol Biochem* 2017;43:1273-1287.
- 32 Dong K, Hao P, Xu S, Liu S, Zhou W, Yue X, Rausch-Fan X, Liu Z: Alpha-Lipoic Acid Alleviates High-Glucose Suppressed Osteogenic Differentiation of MC3T3-E1 Cells via Antioxidant Effect and PI3K/Akt Signaling Pathway. *Cell Physiol Biochem* 2017;42:1897-1906.

- 33 Ahmadi A, Mazooji N, Roozbeh J, Mazloom Z, Hasanazade J: Effect of alpha-lipoic acid and vitamin E supplementation on oxidative stress, inflammation, and malnutrition in hemodialysis patients. *Iran J Kidney Dis* 2013;7:461-467.
- 34 Kim MS, Park JY, Namkoong C, Jang PG, Ryu JW, Song HS, Yun JY, Namgoong IS, Ha J, Park IS, Lee IK, Viollet B, Youn JH, Lee HK, Lee KU: Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. *Nat Med* 2004;10:727-733.
- 35 Chang P, Liu J, Yu Y, Cui SY, Guo ZH, Chen GM, Huang Q, Liu ZG: Alpha-Lipoic Acid Suppresses Extracellular Histone-Induced Release of the Inflammatory Mediator Tumor Necrosis Factor-alpha by Macrophages. *Cell Physiol Biochem* 2017;42:2559-2568.
- 36 Nofer JR, Bot M, Brodde M, Taylor PJ, Salm P, Brinkmann V, van Berkel T, Assmann G, Biessen EA: FTY720, a synthetic sphingosine 1 phosphate analogue, inhibits development of atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation* 2007;115:501-508.
- 37 Serbecic N, Beutelspacher SC: Anti-oxidative vitamins prevent lipid-peroxidation and apoptosis in corneal endothelial cells. *Cell Tissue Res* 2005;320:465-475.
- 38 Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I: Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 2004;114:1752-1761.
- 39 Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EB, Mueller-Dieckmann C, Farahifar D, Rossi B, Auberger P, Baeuerle PA, Peyron JF: Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. *Cell* 1996;86:787-798.
- 40 Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM, Jr., Boerwinkle E: Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. *Circulation* 1997;96:4219-4225.
- 41 Deng H, Chu X, Song Z, Deng X, Xu H, Ye Y, Li S, Zhang Q, Sun C, Li Y: MicroRNA-1185 Induces Endothelial Cell Apoptosis by Targeting UVRAG and KRIT1. *Cell Physiol Biochem* 2017;41:2171-2182.
- 42 Hill RA, Pompolo S, Jones ME, Simpson ER, Boon WC: Estrogen deficiency leads to apoptosis in dopaminergic neurons in the medial preoptic area and arcuate nucleus of male mice. *Mol Cell Neurosci* 2004;27:466-476.
- 43 Rautou PE, Leroyer AS, Ramkhalawon B, Devue C, Duflaut D, Vion AC, Nalbone G, Castier Y, Leseche G, Lehoux S, Tedgui A, Boulanger CM: Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration. *Circ Res* 2011;108:335-343.
- 44 Choudhury RP, Lee JM, Greaves DR: Mechanisms of disease: macrophage-derived foam cells emerging as therapeutic targets in atherosclerosis. *Nat Rev Cardiol* 2005;2:309-315.
- 45 Kim J, Kim JS, Park E: Cytotoxic and anti-inflammatory effects of onion peel extract on lipopolysaccharide stimulated human colon carcinoma cells. *Food Chem Toxicol* 2013;62:199-204.
- 46 van Diepen JA, Berbee JF, Havekes LM, Rensen PC: Interactions between inflammation and lipid metabolism: relevance for efficacy of anti-inflammatory drugs in the treatment of atherosclerosis. *Atherosclerosis* 2013;228:306-315.
- 47 Vo TS, Kim SK: Down-regulation of histamine-induced endothelial cell activation as potential anti-atherosclerotic activity of peptides from *Spirulina maxima*. *Eur J Pharm Sci* 2013;50:198-207.
- 48 Straliootto MR, de Oliveira J, Mancini G, Bainy AC, Latini A, Deobald AM, Rocha JB, de Bem AF: Disubstituted diaryl diselenides as potential atheroprotective compounds: Involvement of TrxR and GPx-like systems. *Eur J Pharm Sci* 2013;48:717-725.
- 49 Boardman HM, Hartley L, Eisinga A, Main C, Roque i Figuls M, Bonfill Cosp X, Gabriel Sanchez R, Knight B: Hormone therapy for preventing cardiovascular disease in post-menopausal women. *Cochrane Database Syst Rev* 2015:CD002229.
- 50 Bassuk SS, Manson JE: The timing hypothesis: Do coronary risks of menopausal hormone therapy vary by age or time since menopause onset? *Metabolism* 2016;65:794-803.
- 51 Dudek M, Bilska-Wilkosz A, Knutelska J, Mogilski S, Bednarski M, Zygmunt M, Iciek M, Sapa J, Bugajski D, Filipek B, Wlodek L: Are anti-inflammatory properties of lipoic acid associated with the formation of hydrogen sulfide? *Pharmacol Rep* 2013;65:1018-1024.

- 52 Tang C, Xue HL, Bai CL, Fu R: Regulation of adhesion molecules expression in TNF-alpha-stimulated brain microvascular endothelial cells by tanshinone IIA: involvement of NF-kappaB and ROS generation. *Phytother Res* 2011;25:376-380.
- 53 Kim SR, Bae YH, Bae SK, Choi KS, Yoon KH, Koo TH, Jang HO, Yun I, Kim KW, Kwon YG, Yoo MA, Bae MK: Visfatin enhances ICAM-1 and VCAM-1 expression through ROS-dependent NF-kappaB activation in endothelial cells. *Biochim Biophys Acta* 2008;1783:886-895.
- 54 Gupta SC, Sundaram C, Reuter S, Aggarwal BB: Inhibiting NF-kappaB activation by small molecules as a therapeutic strategy. *Biochim Biophys Acta* 2010;1799:775-787.
- 55 Zhang R, Yin X, Shi H, Wu J, Shakya P, Liu D, Zhang J: Adiponectin modulates DCA-induced inflammation via the ROS/NF-kappa B signaling pathway in esophageal adenocarcinoma cells. *Dig Dis Sci* 2014;59:89-97.
- 56 Ramyaa P, Padma VV: Ochratoxin-induced toxicity, oxidative stress and apoptosis ameliorated by quercetin-modulation by Nrf2. *Food Chem Toxicol* 2013;62:205-216.
- 57 Zhang J, Wang X, Vikash V, Ye Q, Wu D, Liu Y, Dong W: ROS and ROS-Mediated Cellular Signaling. *Oxid Med Cell Longev* 2016;2016:4350965.
- 58 Montezano AC, Burger D, Ceravolo GS, Yusuf H, Montero M, Touyz RM: Novel Nox homologues in the vasculature: focusing on Nox4 and Nox5. *Clin Sci* 2011;120:131-141.
- 59 Serrander L, Cartier L, Bedard K, Banfi B, Lardy B, Plastre O, Sienkiewicz A, Forro L, Schlegel W, Krause KH: NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation. *Biochem J* 2007;406:105-114.
- 60 Bedard K, Krause KH: The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 2007;87:245-313.
- 61 Zhang Y, Qin W, Zhang L, Wu X, Du N, Hu Y, Li X, Shen N, Xiao D, Zhang H, Li Z, Zhang Y, Yang H, Gao F, Du Z, Xu C, Yang B: MicroRNA-26a prevents endothelial cell apoptosis by directly targeting TRPC6 in the setting of atherosclerosis. *Sci Rep* 2015;5:9401.
- 62 Tamir S, Izrael S, Vaya J: The effect of oxidative stress on ERalpha and ERbeta expression. *J Steroid Biochem Mol Biol* 2002;81:327-332.
- 63 Shimizu H, Saito S, Higashiyama Y, Nishijima F, Niwa T: CREB, NF-kappaB, and NADPH oxidase coordinately upregulate indoxyl sulfate-induced angiotensinogen expression in proximal tubular cells. *Am J Physiol Cell Physiol* 2013;304:C685-692.