Abstract. Endothelial cell injury in vascular arterial walls plays a crucial role in the pathological process of atherosclerosis. Pterostilbene, a stilbenoid chemically related to resveratrol, has anti-inflammatory, anti-apoptosis and antioxidant properties. However, the underlying mechanisms mediated by pterostilbene in regards to endothelial cell injury in vascular arterial walls are not fully understood. The purpose of the present study was to investigate the benefits of pterostilbene in a rat model of atherosclerosis. The possible mechanism of pterostilbene was also analyzed in regards to endothelial cell injury in vascular arterial walls in vitro. A rat model of atherosclerosis was established using endothelial injury of the iliac arteries. CCK-8 assay, TUNEL, immunofluorescence, western blot analysis and hematoxylin and eosin (H&E) staining were used to analyze the role of pterostilbene in the pathological processes of atherosclerosis. In vivo results showed that pterostilbene decreased cholesterol (CHO), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) in plasma and attenuated interleukin (IL)-1, tumor necrosis factor (TNF)-α and IL-6 and oxidative stress injury in serum in the experimental animals. Pterostilbene treatment reduced atherogenesis, aortic plaques, macrophage infiltration and apoptosis of vascular arterial walls in the atherosclerosis rat model. In vitro assay demonstrated that pterostilbene administration increased viability of the endothelial cells, attenuated oxidative stress injury and apoptosis of endothelial cells. The results found that pterostilbene regulated endothelial cell apoptosis via the Nrf2-mediated TLR-4/MyD88/NF-κB pathway. In conclusion, data from the present study revealed that pterostilbene protects rats against atherosclerosis by regulation of the Nrf2-mediated TLR-4/MyD88/NF-κB pathway.

Introduction

Atherosclerosis is a chronic inflammatory disease of the vascular wall associated with lipid deposition and plaque fibrosis (1-3). As a chronic inflammatory disease, the mortality rate caused by atherosclerosis is rising worldwide (4). Pathologically, dysfunction of endothelial cells is a critical event in the pathological process of atherosclerosis, which leads to apoptosis of vascular arterial walls, atherogenesis, aortic plaque and macrophage infiltration (5-7). In addition, atherosclerosis is generally regarded as a lipid-induced chronic inflammation in the vessel wall, which leads to inflammatory lesions and apoptosis of endothelial cells (8-10). Thus, elucidating the possible mechanisms underlying the inflammatory process and apoptotic signal molecular pathways involved in the pathology of atherosclerosis are crucial to understand the occurrence and development of atherosclerosis.

Pterostilbene is a dimethylated analog of resveratrol that exhibits protective ability against atherosclerosis (11). Research indicates that pterostilbene is an anti-inflammatory compound that causes improvement of atherosclerotic plaque macrophages in patients with atherosclerosis (12). Recently, Gao et al (13) found that pterostilbene can protect rats against acute renal ischemia reperfusion injury and inhibit oxidative stress and inflammation via the TLR4/NF-κB signaling pathway. In addition, atherosclerosis is partly mediated by the dysfunction and apoptosis of endothelial cells (14). Meanwhile, the apoptosis of endothelial cells in the artery wall increases atherosclerotic inflammation and plaques in the formation of foam cells and necrotic core along with lipid uptake of macrophages (15-17).
However, understanding the multifactorial process of pterostilbene in mediating the pathological process of atherosclerotic plaques to protect endothelial cells against atherosclerosis have not been well investigated.

The TLR-4/MyD88/NF-kB signaling pathway has been widely investigated in previous studies (18-20). Qi et al suggest that suppression of the TLR4/MyD88 signaling pathway confers a protective effect against renal ischemia/reperfusion injury (21). Inactivation of the TLR4/MyD88/NF-kB signaling pathway was found to exhibit potent effects against alcoholic liver fibrosis (22). In addition, Yao et al demonstrated that downregulating the TLR4/MyD88 signaling pathway reduced lipopolysaccharide-induced inflammatory liver injury (23). Furthermore, research also indicates that suppression of the TLR4/MyD88 signaling pathway exerts a nephroprotective effect against LPS-induced inflammatory renal injury, which provides novel insights into the mechanisms of this therapeutic candidate for the treatment of inflammatory injury (24). Thus, the TLR-4/MyD88/NF-kB signaling pathway may be considered as a new potential therapeutic option for the treatment of inflammatory disease.

In the present study, the potential therapeutic effects of pterostilbene were investigated where the possible underlying mechanism in endothelial cells in a rat model of atherosclerosis was explored. The anti-inflammatory, antioxidant and anti-apoptotic abilities of pterostilbene in endothelial cells in the vascular arterial walls were also analyzed in the rat model of atherosclerosis. These results revealed that pterostilbene treatment decreased the inflammation, oxidative stress and apoptosis of arterial endothelial cells and thus prevented rats against the formation atherosclerotic plaque.

**Materials and methods**

*Establishment of the rat model of atherosclerosis.* The use of experimental animals in the present study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA) (25). The animal use protocol was approved by the Committee on the Ethics of Animal Experiments of the Second Affiliated Hospital of Nanchang University (Nanchang, Jiangxi, China). Male, 8-week-old Sprague-Dawley rats with initial body weight of 300-320 g (n=26) were purchased from the Second Affiliated Hospital of Nanchang University. All animals were housed with a 12-h light-dark cycle, at 23±1˚C and 50±5% humidity. All rats had free access to food and water. All surgeries were performed under anesthesia with pentobarbital (40 mg/kg), and efforts were made to minimize suffering of the rats. The rats were fed a 2.5% cholesterol diet for 8 weeks as described previously (26). The rats were randomly divided into three experimental groups: healthy group (n=6); group that received phosphate-buffer saline (PBS) treatment (n=10); group that orally received pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene, C₁₆H₁₂O₃, ≥99%, purity; 10 mg/kg/day; Great Forest Biomedical, Hangzhou, China; n=10) treatment once a day. All treatments were continued with a regular diet for 4 weeks.

*Biochemical assays.* After 4 weeks of treatment, rats in each group were injected with pentobarbital for anesthesia, following which 2 ml blood was taken from the retroorbital venous plexus and were then sacrificed. Serum samples were obtained using centrifugation at 8,000 x g for 10 min at 4˚C. The concentrations of IL-6 (cat. no. R6000B), IL-1β (cat. no. RLB00) and TNF-α (cat. no. RTA00) were determined using enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Bio-Rad Laboratories, Inc.). Superoxide dismutase (SOD), catalase (CAT), heme oxygenase-1 (HO-1), malondialdehyde (MDA), myeloperoxidase (MPO), cholesterol (CHO), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels were evaluated using a serum biochemical autoanalyzer (Hitachi 7600 Modular Chemistry Analyzer; Hitachi Ltd.) according to the manufacturer's instructions.

*Histological examination.* After sacrifice, half of the aortic arch samples were obtained, fixed in 10% paraformaldehyde, dehydrated by grading ethanol, paraffin embedded and cut into 4-μm sections. The sections then underwent antigen retrieval using eBioscience™ IHC Antigen Retrieval Solution (cat. no. 00-4955-58, Invitrogen; Thermo Fisher Scientific, Inc.). Tissue sections were stained with hematoxylin and eosin (H&E) and then stained with hematoxylin and 0.5% Oil Red O (Sigma-Aldrich; Merck KGaA) for 1 h at 37˚C. The histopathological and histomorphometric images were evaluated by three pathologists and captured using Image-Pro Plus software v2.0 (Media Cybernetics).

*TUNEL assay.* The apoptosis of cells in tissues was analyzed using terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick-end labeling (TUNEL) staining kit (Roche). Tissue sections were stained with TUNEL for 2 h at room temperature and analyzed using a commercial TUNEL staining kit (Roche) according to the manufacturer's protocol, following which they were placed in hematoxylin for 2 min at 37˚C. The sections were then washed for 2 min with PBS, dipped in 95% ethanol, absolute ethanol I-III for 3.5 min, xylene I-III for 3.5 min and sealed with neutral resins. Tissues sections were captured using a light microscope (Olympus Corporation) at magnification, x100.

*Cell culture.* Endothelial cells were purchased from BeNa Collection Culture (Beijing, China) and cultured in OptiMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA) and 100 µg/ml streptomycin (Sigma-Aldrich; Merck KGaA) at 37˚C in 5% CO₂. Cells were treated with pterostilbene (1.0 mmol/l for 12 h at 37˚C. All experiments were performed in triplet.

*Cell viability assay.* Viability of endothelial cells was analyzed using the Cell Counting Kit-8 (CCK-8; Sigma-Aldrich; Merck KGaA). Briefly, endothelial cells at 1x10⁵ cells/ml density were seeded into six-well plates, along with the addition of 0.2% H₂O₂ for 4 h and then incubated with pterostilbene (1.0 mmol/l) for 12 h at 37˚C. A total of 10 µl CCK-8 solution was added into the cells which were then cultured for 30 min at 37˚C. Cell viability was determined at 450 nm absorbance using a microplate reader (Bio-Rad Laboratories, Inc.).
Gene knockdown. Endothelial cells (1x10^5/well) were seeded into a 6-well plate. After 24 h, the cells were transfected with 40 pmol siRNA-Nrf2 (5'-GAGUAGAGGCUAGGAA AAAACU-3'; Shanghai GenePharma Co., Ltd.) or siRNA-NC (5'-GACGAGCGCACGUCCAUU-3'; Shanghai GenePharma Co., Ltd.) using Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cells were used for further analyses after a 72-h transfection.

Reactive oxygen species (ROS) activity. Endothelial cells were cultured in a 6-well plate at a density of 1.0x10^5/ml. Cells were treated with pterostilbene (1.0 mmol/l) for 12 h at 37°C. Then the cells were rinsed with PBS, and incubated with 10 µM 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA, Molecular Probes; Thermo Fisher Scientific, Inc.) for 30 min at 37°C. Images of the cells were captured using a Zeiss Inverted Microscope (magnification, ×100; Carl Zeiss). Fluorescence activity was measured using a fluorometric plate reader (BMG Labtech GmbH) at excitation 530 nm/emission 485 nm.

Western blot analysis. The treated endothelial cells (1x10^5) were lysed in RIPA buffer (Bio-Rad Laboratories, Inc.) and the lysates were centrifuged at 10,000 x g for 10 min at 4°C. Protein concentrations were measured using a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) and a total of 20 µg protein was electrophoresed using 10% SDS-PAGE followed by transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore). The membranes were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA), and incubated with the following primary antibodies: SOD (dilution 1:1,000, cat. no. ab13534, Abcam), CAT (dilution 1:1,000, cat. no. ab16731, Abcam), HO-1 (dilution 1:1,000, cat. no. ab13243, Abcam), Nrf2 (dilution 1:1,000, cat. no. ab62352, Abcam), TLR-4 (dilution 1:1,000, cat. no. ab13556, Abcam), MyD88 (dilution 1:1,000, cat. no. ab2064, Abcam), NF-kB (dilution 1:1,000, cat. no. ab131546, Abcam), phosphorylated NF-kB (pNF-kB, dilution 1:1,000, cat. no. ab20803, Abcam), IL-1 (dilution 1:2,000, cat. no. ab200478, Abcam), TNFα (dilution 1:2,000, cat. no. ab6671, Abcam), IL-17 (dilution 1:2,000, cat. no. ab79056, Abcam) and β-actin (dilution 1:2,000, cat. no. ab8226, Abcam) for 12 h at 4°C. Protein was then incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:5,000, cat. no. ab205718, Abcam) for 2 h at 37°C. The bands of proteins were observed with an enhanced chemiluminescence (ECL) substrate kit (Beyotime Institute of Biotechnology). Quantitative expression of proteins was quantified using ImageJ software (v4.6.2; National Institutes of Health, Bethesda).

Flow cytometry. Apoptosis of endothelial cells was analyzed using flow cytometry. The treated endothelial cells (1x10^5) were stained with 5 µl Annexin V-FITC and 10 µl propidium iodide (PI) solution for 30 min at 4°C in the dark. The number of apoptotic cells was examined by FACS (BD Biosciences). Data acquisition and analysis were evaluated using a BD Flow Cytometer v1.0 (BD Biosciences) with NovoExpress® software v1.2 (ACEA Biosciences, Inc.).

Statistical analysis. All data are reported as means ± SD. Statistical analysis was analyzed by the Student t test or one-way ANOVA followed by Tukey's test using SPSS software, v19.0 (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of pterostilbene on body weight, blood pressure and lipid metabolism in the experimental rats. The effects of pterostilbene on body weight, blood pressure and lipid metabolism were analyzed in the rat model of atherosclerosis. The results demonstrated that pterostilbene increased the body weight and reduced blood pressure in the rats with atherosclerosis when compared to the PBS group (Fig. 1A and B). Pterostilbene treatment decreased CHO, HDL-C, TC, and LDL-C levels in the plasma of the rats with atherosclerosis rat compared to these levels in the PBS group (Fig. 1C).

Effect of pterostilbene on oxidative stress injury in the experimental rats. The antioxidative activity of pterostilbene was investigated in the rats with atherosclerosis. Serum levels of SOD, CAT and HO-1 were determined using corresponding kits. The results revealed that the levels of SOD, CAT and HO-1 were markedly upregulated in the pterostilbene-treated rats when compared with the PBS group (Fig. 2A). Conversely, the MDA and MPO levels in serum were significantly decreased by pterostilbene treatment when compared to these levels in the PBS group (Fig. 2B). These results indicated that pterostilbene can decrease vascular injury-induced oxidative stress.

Effect of pterostilbene on inflammation and pathological features in the experimental rats. The anti-inflammatory efficacy of pterostilbene was investigated in the rat model of atherosclerosis. As shown in Fig. 3A, administration of pterostilbene decreased serum IL-1, TNF-α and IL-6 levels in the experimental animals compared to these levels in the PBS group. Pterostilbene treatment reduced atherogenesis and aortic plaques (Fig. 3B). Histological analysis demonstrated that pterostilbene treatment markedly decreased macrophage infiltration and apoptosis of vascular arterial walls in the atherosclerosis rat model (Fig. 3C and D).

Effects of pterostilbene on cell viability and apoptosis in endothelial cells. We next investigated the benefits of pterostilbene in endothelial cells in vitro. The results demonstrated that pterostilbene increased the viability of endothelial cells when compared to the control (Fig. 4A). Administration of pterostilbene significantly attenuated oxidative stress injury as determined by levels of SOD, CAT and HO-1 and significantly reduced apoptosis of the endothelial cells as compared to the control (Fig. 4B and C). Pterostilbene treatment also significantly deceased IL-1, TNF-α, and IL-6 expression in the endothelial cells when compared to the control (Fig. 4D). As illustrated in Fig. 4E, pterostilbene reduced ROS activity in the endothelial cells compared to the control group.

Pterostilbene inhibits endothelial cell apoptosis by the Nrf2-mediated TLR-4/MyD88/NF-kB pathway. We finally investigated the possible mechanism underlying the pterostilbene-mediated inhibition of endothelial cell apoptosis. Western blot analysis showed that pterostilbene increased Nrf2 and...
decreased TLR-4, MyD88, NF-κB expression and NF-κB phosphorylation in the endothelial cells (Fig. 5A). Nrf2 knockdown (siR-Nrf2) increased and canceled pterostilbene-mediated regulation of TLR-4, MyD88, NF-κB expression and NF-κB phosphorylation in the endothelial cells (Fig. 5B). The results demonstrated that pterostilbene-mediated inhibition of endothelial cell apoptosis was abolished via siR-Nrf2 (Fig. 5C).

Discussion

It is widely recognized that apoptosis of endothelial cells and inflammatory lesions in artery blood vessels promote plaque necrosis, which further lead to plaque instability, thrombosis and atherosclerosis (27). In the present study, we reported the therapeutic effects of pterostilbene in a rat model of atherosclerosis. Lipid metabolism, inflammatory cytokines, body weight, blood pressure and improvement of pathological features were investigated in atherosclerotic rats after a 4-week pterostilbene treatment. Treatment with pterostilbene decreased serum lipid metabolism and attenuated hyperlipidemia and aortic inflammation in the rats with atherosclerosis, as well as inhibition of the apoptosis of the endothelial cells, ultimately improving atherosclerosis. The present study elucidated a novel mechanism explaining the protective effects of pterostilbene on the apoptosis of endothelial cells in vascular arterial walls, and provides insight into the potential mechanisms and strategies for anti-atherosclerosis treatment. Notably, the findings of the present study demonstrated that pterostilbene reduced endothelial cell apoptosis by the Nrf2-mediated TLR-4/MyD88/NF-κB pathway.

Clinically, atherosclerosis is characterized by dysfunction in lipid homeostasis and endothelial cells in vascular arterial walls, slow metabolism, impairment of signaling pathways and increased levels of inflammatory cytokines (28-30). The anti-adipogenesis mechanism of pterostilbene has been
identified to activate heme oxygenase-1 in 3T3-L1 cells (31). Here, we used a rat model of atherosclerosis to specify the regulatory effects of pterostilbene on lipid homeostasis and found that pterostilbene decreased high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels in the plasma of the atherosclerotic rats, which further led to improvement in body weight and blood pressure. A previous study found that pterostilbene decreased cardiac oxidative stress and inhibited high fat-induced atherosclerosis inflammation (32). We found that pterostilbene attenuated inflammatory cytokines as evidenced by decreased levels of interleukin (IL)-1, tumor necrosis factor (TNF)-α and IL-6, which further decreased endothelial cell apoptosis and oxidative stress injury. However, the potential

Figure 3. Effect of pterostilbene treatment on inflammation and histological features in the experimental rats. (A) Pterostilbene treatment decreased serum IL-1, TNF-α, and IL-6 levels in the rats with atherosclerosis. *P<0.01 vs. the PBS-treated group. NS, not significant. (B) Pterostilbene treatment reduced atherogenesis and aortic plaques. Scale bars: upper images, 50 µm; lower images, 100 µm. (C) Effects of pterostilbene on the apoptosis of vascular arterial wall cells in the rat model of atherosclerosis. Arrow indicates the locations of apoptotic cells in the vascular arterial walls. Red arrow indicates apoptosis of vascular arterial wall cells. (D) Pterostilbene treatment decreased macrophage infiltration. Black arrow indicates macrophage infiltration. IL, interleukin; TNF-α, tumor necrosis factor α.
molecular mechanisms underlying the anti-atherogenic effects of pterostilbene require further elucidation in subsequent research.

Pterostilbene was found to increase protein expression of Nrf2 in cardiac tissues, which may account for the prevention of cardiac oxidative stress and inflammation in
fructose-fed rats (32). Chen et al (33) showed that pterostilbene protects against uremia serum-induced endothelial cell damage via activation of the Keap1/Nrf2/HO-1 signaling pathway. The present study found that treatment with pterostilbene increased Nrf2, while Nrf2 knockdown canceled the pterostilbene-mediated inhibition of endothelial cell apoptosis. The TNF-α mediated-NF-κB signaling pathway is involved in endothelial cell apoptosis and the progression of atherosclerosis (34). Hosseini et al demonstrated that suppression of the TLR-4-MyD88 signaling pathway is essential for atheroprotection by reducing lesion inflammatory cytokines TNF-α, IL-1β, and IL-18 (35). Our data showed that therapeutic strategy of pterostilbene protected animals against atherosclerosis via the Nrf2-mediated TLR-4/MyD88/NF-κB pathway. Moreover, pterostilbene treatment significantly increased the antioxidant molecules including catalase (CAT),
Inhibition of local macrophage growth via downregulation of the TLR-4/MyD88/NF-κB pathway (36). Enhancing the Nrf2 pathway was found to protect against LPS-induced sepsis via the TLR4/MyD88/IKκB pathway (37). These findings are consistent with the fact that TLR-4/MyD88/NF-κB expression is downregulated by Nrf2 in pterostilbene-treated endothelial cells that respond to anti-inflammatory and anti-apoptotic efficacy. However, this study analyzed the total Nrf2 expression, but did not determine the nuclear and cytoplasmic fractions in the endothelial cells. Thus, further investigation needs to differentiate the nuclear and cytoplasmic expression of Nrf2 in endothelial cells after treatment with pterostilbene in future research.

In conclusion, data in the present study demonstrated that pterostilbene exhibited multifaceted effects on lipid metabolism, reduced inflammation, and decreased endothelial cell apoptosis in a rat model of atherosclerosis. Administration of pterostilbene activated the Nrf2 signaling pathway in endothelial cells by altering downstream expression of the TLR-4/MyD88/NF-κB pathway, which is involved in antioxidant mechanisms and apoptosis. In addition, association between pterostilbene and the pathological features of atherosclerosis was discovered in the present study, which broadens the clinical implications of pterostilbene for the treatment of cardiovascular disease. However, additional molecular mechanisms mediated by pterostilbene warrant further investigate for targeting atheromatous plaque.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XWX and WHL performed the experiments. KHZ analyzed data. WMZ designed the study and wrote this manuscript. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Third Affiliated Hospital of Nanchang University (approval no. ZKY20190044, Nanchang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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