Paeonol Inhibits Oxidized Low Density Lipoprotein-Induced Monocyte Adhesion to Vascular Endothelial Cells by Inhibiting the Mitogen Activated Protein Kinase Pathway

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Atherosclerosis is a chronic inflammatory disease characterized by increased expression of adhesion molecules, which contribute to monocytes adhesion to vascular endothelial cells (VECs). Paeonol, an active compound isolated from cortex Moutan, has been shown to have therapeutic effects on atherosclerotic animals. The present study aims to investigate whether paeonol can inhibit monocyte adhesion to vascular endothelial cells induced by oxidized Low-Density Lipoprotein (ox-LDL) and its possible therapeutic molecular mechanism. Exposure to ox-LDL (50, 100 µg/mL) induced damaged to VECs leading to decreased survival rates (p<0.01). Paeonol (7.2–18.0 µM) partially restored survival and reduced lactate dehydrogenase (LDH) release in VECs in a concentration-dependent manner (p<0.01). Adhesion of monocytes to VECs was dramatically prevented by paeonol at 21.6 and 25.2 µM (p<0.01). In addition, paeonol (14.4–21.6 µM) repressed the expression of vascular cell adhesion molecule-1 (VCAM-1) and lowered the levels of phosphor-c-Jun N-terminal kinase (P-JNK)1/2, phosphor-extracellular signal-regulated kinase (P-ERK)1/2 and P-p38 in a dose-dependent manner. The molecular effects of paeonol were more pronounced when accompanied with mitogen activated protein kinases (MAPKs) inhibitors. These data suggest that paeonol (10.8–25.2 µM), at certain concentrations, prevents monocyte adhesion to VEC induced by ox-LDL, probably by means of blocking one or more target proteins on MAPKs signaling pathway. These results indicate that paeonol has potential protective effects on the development of atherosclerosis.

Key words paeonol; oxidized Low-Density Lipoprotein; vascular endothelial cell; monocyte; mitogen activated protein kinase

Atherosclerosis (AS) is a chronic disease which causes high morbidity and mortality as a result of cardiovascular disorders. Many factors can lead to AS such as high cholesterol, hypertension and infections, giving rise to extensive endothelial injury. Oxidized Low-Density Lipoprotein (ox-LDL) plays an important role in the progression of atherosclerosis. Ox-LDL can reduce endothelial regeneration and activate endothelial adhesive properties. In addition, ox-LDL initiates monocyte invasion in subendothelial space. Certainly, ox-LDL can enhance the expression of vascular cell adhesion molecule-1 (VCAM-1), which assists monocytes adhesion to endothelial cells and initiates the formation of atherosclerotic lesions.

Mitogen activated protein kinase (MAPK) pathway is a key signal transduction pathway and is associated with many inflammatory diseases. MAPK pathways are activated by many cytokines and growth factors, which are produced under a state of stress and infection. In mammalian cells, MAPK family members are comprised of four members including extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 and big MAP kinase 1 (BMK1)/ERK5. Previous studies have indicated that cell adhesion is mainly dependent on ERK 1/2 and p38. Therefore, the development of atherosclerosis may be associated with the MAPK pathway. Paeonol (Fig. 1), an active compound isolated from cortex Moutan, is widely used in clinical diseases such as atopic dermatitis and hyperlipemia. Our previous studies have shown that paeonol has many pharmacological activities. In vivo, paeonol prevents experimental AS in quail and is protective against vascular endothelial cells (VECs) from hyperlipidemia. In vitro, paeonol reduces the formation of ox-LDL lesions by inhibiting lipid peroxidation and down-regulating VCAM-1 expression against tumor necrosis factor alpha (TNF-α). These molecular mechanisms may be due to paeonol antioxidative, anti-inflammatory and antithrombotic activities. In short, the current study was carried out to evaluate the potential protective effect of paeonol on AS and determine its possible mechanisms on MAPK pathways.

MATERIALS AND METHODS

Chemicals The compound paeonol (98% purity) was obtained from Baicao Plants Biotech Co., Ltd. (Anhui, China), dissolved in dimethyl sulfoxide (DMSO) of which final concentration was less than 0.1%. Rabbit polyclonal antibodies for phosphor-ERK1/2, ERK1/2, phosphor-JNK1/2, JNK1/2, phosphor-p38 MAPK and p38MAPK were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). SP600125 and PD98059 were bought from Beyotime institute of Biotech (Nanjing, China). SB203580 was purchased from Haoran

Fig. 1. The Chemical Structure of Paeonol

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Biotech Co., Ltd. (Shanghai, China). VCAM-1 monoclonal antibody was purchased from Boshide Biotech Co., Ltd. (Wuhan, China). Rose Bengal was purchased from Solabao (Beijing, China). Fetal bovine serum (FBS) was purchased from Hao Yang Biological Products Co., Ltd. (Tianjin, China). The amount of endotoxin contained in all drugs and reagents complied with requirements.19

Animals Healthy Sprague-Dawley rats (160±10 g) were used in this study. The experiment was conducted in accordance with animal welfare at Anhui University of Traditional Chinese Medicine, China.

Vascular Endothelial Cell Culture Vascular endothelial cells were isolated from rat thoracic aortas as previously described20 and cultured using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% heat-inactivated FBS and penicillin/streptomycin. Trypan blue (4 mg/mL) was used to determine cell viability of more than 95 percent. In all experiments cells were used up to passage 3–5 and serum-free starved for 24 h to synchronize growth cycles.

Monocyte Culture Blood from abdominal aorta of Sprague-Dawley rats was centrifuged at 1600×g for 10 min. Then cells were suspended with lymphocyte separation solution and centrifuged at 600×g for 15 min. Monocytes were washed with phosphate buffered saline (PBS) and DMEM and centrifuged at 240×g for 6 min. During all studies monocyte concentrations were adjusted to 1×10⁶ cells/mL with DMEM.21

Ox-LDL Preparation Fresh abdominal aortic blood from healthy rats was coagulated and centrifuged at 1700×g for 10 min. Citrate buffer (1 mL) was added to serum (100 µL) and the precipitate was dissolved in phosphate buffer. LDL concentration was determined according to Lowry method22 and adjusted to 3.2 mg/mL with PBS. LDL was incubated with fresh CuSO₄ (160 µmol/L) at 37°C for 15 h and an equal volume of ethylene diaminetetraacetic acid (EDTA)-Na₂ was added to terminate reaction. The degree of oxidation of LDL was detected by the MDA Kit. Ox-LDL concentration was determined by the MDA Kit. Ox-LDL concentration was determined according to Lowry method similar as before. It was filtered with 0.22 µm membrane and stored from light at 4°C.

Survival Rate Analysis of Vascular Endothelial Cells Vascular endothelial cells were grown to confluence in DMEM and seeded into 96-well culture plates. Once grown to confluence in DMEM, VEC was cultured with monocytes at 37°C for 1 h. Afterward, VEC was cultured with monocytes at 37°C for 1 h. Non-adhered monocytes were aspirated before disclosed to Rose-Bengal (0.25%, pH 7.3). Absorbance (A) values which represented adhesion process were measured by microplate reader at 570 nm wavelength.23

Determination of VCAM-1 Expression Sterilized cover slips on which VEC would be seeded were placed in 6-well plates. Once grown to confluence in DMEM, VEC was serum-free starved for 24 h and treated with paenol of 14.4, 18.0, 21.6 µM for 24 h before incubated with 100 µg/mL ox-LDL (final concentration) for 3 h. Afterward, VEC was cultured with monocytes at 37°C for 1 h. Non-adhered monocytes were aspirated before disclosed to Rose-Bengal (0.25%, pH 7.3). Absorbance (A) values which represented adhesion process were measured by microplate reader at 570 nm wavelength.23

Analysis of MAPKs Expression Using Western Blotting Vascular endothelial cells were synchronized and treated with paenol of 14.4, 18.0, 21.6 µM for 24 h before incubated with 100 µg/mL ox-LDL (final concentration) for 3 h. To further determine the effect of paenol on MAPKs pathway, another three groups of VECs were pretreated with the specific JNK inhibitor SP600125 (40 nM), p38 inhibitor SB203580 (60 nM) and ERK inhibitor PD98059 (25 µM) 30 min before paenol exposure. Protein concentrations in VECs was quantified by bicinchoninic acid (BCA) Protein Assay Kit and electrophoresis on 12% SDS-polyacrylamide gel on which proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked for 3 h at room temperature. The PVDF membranes were incubated with primary antibodies (1:1000 overnight and then incubated with secondary antibodies (1:3000) for 2 h. The gel images were acquired through Amersham ImageMaster VDS-CL.

Statistical Analysis Results are presented as mean± S.E.M. for three or more independent experiments. Statistical significance among multiple groups was analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test for comparison of several groups in SPSS11.5 software package. p<0.05 was considered statistically significant.

RESULTS

Quantification of ox-LDL LDL concentration was detected at a final concentration of 5.8 mg/mL. Malondialdehyde (MDA) concentrations were elevated from 0.803±0.0725 (nmol/mL) to 15.956±2.4199** (nmol/mL) after LDL was oxidized. These results indicate that LDL was effectively oxidized (p<0.01), which is consistent with previously reported studies.25,26 The concentration of ox-LDL was 0.8 mg/mL.

Paenol Increases Survival Rate of Vascular Endothelial Cells Normal vascular endothelial cells were characterized with typical cobblestone arrangements such as polygon formations, tightly packed, clear nucleus and smooth edges. However, ox-LDL brought about obvious morphological changes such as shrinkages, cracks among cells and intracellular vacuoles. Compared with control group, ox-LDL of...
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50 µg/mL drastically lowered cell survival rates ($p<0.01$), while ox-LDL induced cell death was ameliorated by paeonol (7.2–18.0 µM) in dose-dependent manner (Fig. 2A).

Additionally, compared with the control group, ox-LDL of 50 µg/mL increased LDH release ($p<0.01$). LDH release was reversed by paeonol (7.2–18.0 µM) in dose-dependent manner ($p<0.01$) (Fig. 2B). These results indicate that paeonol can protect VECs from ox-LDL induced cell death.

Paeonol Prevents Monocytes Adhesion to Vascular Endothelial Cells Ox-LDL promoted monocytes adhesion to vascular endothelial cells and the maximum adhesion took place at 3h of 100 µg/mL (Fig. 3). Pretreatment with paeonol (10.8–25.2 µM) significantly reduced the adhesion rate and the greatest inhibitory effect appeared at 24h of 21.6 and 25.2 µM of paeonol ($p<0.01$) (Fig. 4).

Paeonol Decreases Expression of VCAM-1 Ox-LDL (100 µg/mL) resulted the increased expression of VCAM-1 protein in vascular endothelial cells. Immunohistochemistry indicated that vascular endothelial cells were positive if they stained yellowish brown granules within the nucleus. Paeonol (14.4–21.6 µM) not only decreased positive expression of VCAM-1, but also significantly decreased the number of brown nuclear, which suggested paeonol could inhibit the expression of VCAM-1 induced by ox-LDL.

Paeonol Reduces Phosphorylation of p38, JNK1/2 and ERK1/2 To explore the possible mechanism of paeonol against the adhesion process, we investigated the effect of paeonol on p38, JNK1/2, and ERK1/2 phosphorylation in VECs. As shown in Fig. 6, ox-LDL showed no alteration in p38, JNK1/2, and ERK1/2 expression. However, ox-LDL significantly induced the phosphorylation of p38, JNK1/2 and ERK1/2 ($p<0.01$). Preincubation of VECs with paeonol resulted in a significant decrease in the phosphorylation of p38, JNK1/2 and ERK1/2 compared with ox-LDL ($p<0.01$). Paeonol (21.6 µM) accompanied by SB203580, SP600125 and PD98059 decreased the phosphorylation of p38, JNK1/2 and ERK1/2 compared to the paeonol (21.6 µM) treated group.
Fig. 5. Effect of Paeonol on Expression of VCAM-1 in Vascular Endothelial Cells

Vascular endothelial cells were treated with paeonol (14.4, 18.0, 21.6 µM) for 24 h before being stimulated with ox-LDL (100 µg/mL) for 3 h. (A) Control; (B) ox-LDL (100 µg/mL); (C) paeonol (14.4 µM); (D) paeonol (18.0 µM); (E) paeonol (21.6 µM). Results were shown as mean±S.E.M. from three independent determinations. **p<0.01 compared with control group; #p<0.05; ##p<0.01 compared with ox-LDL group.

Fig. 6. Effect of Paeonol on Phosphorylation of p38, JNK1/2 and ERK1/2 in Vascular Endothelial Cells

Vascular endothelial cells were treated with paeonol (14.4, 18.0, 21.6 µM) for 24 h before being stimulated by ox-LDL (100 µg/mL) for 3 h. Results were shown as mean±S.E.M. from three experiments with triplicate determinations. **p<0.01 compared with control group; #p<0.05; ##p<0.01 compared with ox-LDL group; ▲p<0.05 compared with paeonol (21.6 µM) group.
Ox-LDL is a compound that stimulates endothelial cells to produce cytokines and growth factors. Ox-LDL also inhibits nitric oxide (NO) production and promotes reactive oxygen species (ROS) generation. These factors all promote the occurrence and development of AS. This study provides data that indicates that ox-LDL leads to injury and dysfunction of vascular endothelial cells, which is an important and selective permeability barrier of the blood vessel wall. This barrier process serves a critical role at the early stages of AS. Paeonol has protective effect on VEC from being damaged and results in increased VEC viability and decreases LDH release.

Ox-LDL increased the expressions of VCAM-1, an intercellular adhesion molecule-1 (ICAM-1) in the vascular endothelium, which promotes monocyte adhesion and proliferation. Our study indicates that paeonol prevents monocytes adhesion to vascular endothelial cells induced by ox-LDL in dose-dependent manner. Paeonol, directly or indirectly, also alters the expression of adhesion molecules and reduces leukocytes infiltration into vascular endothelium. Paeonol may lead to decreased expression of adhesion molecules that may lead to a novel therapeutic to reduce atherosclerosis.

When MAPK are activated, these proteins rapidly translocate to nucleus where they combine with target genes and up-regulate their expressions. In endothelial cells damaged by ox-LDL, MAPK proteins will overexpress cell adhesion molecules through MAPKs pathway. This study indicates that ox-LDL significantly increased phosphorylation of ERK1/2, JNK1/2, p38 in vascular endothelial cells. Surprisingly, paeonol produced a balanced inhibitory effect on all three pathways. Additionally, all three inhibitors, SB203580, SP600125 and PD98059, inhibited phosphorylation of ERK1/2, JNK1/2 and p38. The expression levels of ERK1/2, JNK1/2 and p38 were not significantly altered among different treatment groups.

Multiple studies have indicated that there are significant interactions between the multiple MAPK signaling pathway proteins. This premise is supported by the present research, indicating that paeonol may decrease the protein levels of VCAM-1 by inhibiting one or more of the three MAPK pathways. Future studies will focus on the upstream cellular effects of paeonol and addition proteins involved in the MAPK pathway.

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REFERENCES

23) Wang DX, Li XP. Inhibitory effect of chitosan on TNF-induced


