Paeonol suppresses lipid accumulation in macrophages via upregulation of the ATP-binding cassette transporter A1 and downregulation of the cluster of differentiation 36

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Abstract. Paeonol, a potent antioxidant isolated from cortex moutan, possesses athero-protective activity, yet the detailed mechanisms are not fully investigated. This study was conducted to explore the role of paeonol and its underlying mechanisms in RAW264.7 macrophages and apolipoprotein E-deficient ($ApoE^{-/-}$) mice. Paeonol treatment significantly attenuated intracellular lipid accumulation in macrophages, which may be the result of decreased oxidized low-density lipoprotein (ox-LDL) uptake and increased cholesterol efflux. Additionally, paeonol markedly inhibited the mRNA and protein expression of the cluster of differentiation 36 (CD36) by decreasing nuclear translocation of c-Jun [a subunit of activator protein-1 (AP-1)]. Moreover, paeonol upregulated the protein stability of ATP-binding cassette transporter A1 (ABCA1) by inhibiting calpain activity, while ABCA1 mRNA expression was not altered. Furthermore, small hairpin RNA (shRNA) targeting haem oxygenase-1 (HO-1) inhibited the paeonol-mediated beneficial effects on the expression of c-Jun, CD36, ABCA1, calpain activity and lipid accumulation in macrophages. Accordingly, paeonol retarded the progress of atherosclerosis in $ApoE^{-/-}$ mice and modulated the expression of CD36 and ABCA1 in aortas similarly to that observed in macrophages. These results indicate that paeonol provides protective effects on foam cell formation by a novel HO-1-dependent mediation of cholesterol efflux and lipid accumulation in macrophages.

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Introduction

Atherosclerosis, a major cause of sudden cardiac death, acute myocardial infarction, and unstable angina pectoris (1), is a chronic arterial disease characterized by lipid deposition and inflammation in the vessel wall (2). Macrophage-derived foam cells play a key role in the development of atherosclerosis. Foam cell formation is primarily caused by impaired cholesterol efflux or uncontrolled uptake of oxidized low-density lipoprotein (ox-LDL) in macrophages (3). There are several classes of scavenger receptors (SRs) on macrophage membrane, in which class A (SR-A) and B (SR-B) attract more attention (4). For instance, SR-A and the cluster of differentiation 36 (CD36) are predominantly responsible for the uptake of ox-LDL (5). In contrast, efflux of intracellular lipid occurs primarily through reverse cholesterol transporters (RCTs) including ATP-binding cassette transporter A1 (ABCA1), ABCG1, and SR-B type I (SR-BI) (6,7). Hence, foam cell formation is mainly mediated by these RCTs and SRs. Ample evidence has indicated that regulation of these SRs or RCTs by antioxidants inhibits the lipid accumulation in foam cells, retarding the progress of atherosclerosis (3,8).

Paeonol (Fig. 1), an active compound isolated from cortex moutan, possesses anti-proliferative properties and apoptosis-inducing activity in different cancer cell types (9-12). Besides its anticancer effect, paeonol has been reported to display several beneficial effects in cardiovascular system including vascular dilation (13), reduction of platelet aggregation (14) and improvement of ischemia reperfusion injury in animals (15). Moreover, paeonol is effective in treatment of atherosclerosis by inhibiting inflammation (16), decreasing thromboxane A2 and free radical formation (17,18), protecting vascular endothelial cells (19), and regulating lipid metabolism (20). Nevertheless, studies on the effects and molecular mechanism by which paeonol mediates lipid accumulation

in macrophage-derived foam cells are not well documented. Additionally, various genetic population studies emphasized the importance of haem oxygenase-1 (HO-1) expression in the protection against human atherosclerotic lesions (21). However, whether HO-1 is involved in the anti-atherogenic effect of paeonol on foam cell formation remains to be investigated.

In the present study, we investigated the effects of paeonol on atherosclerosis and the underlying mechanisms in RAW264.7 cells (mouse macrophage cell line) and apolipoprotein E-deficient ($ApoE^{-/-}$) mice. We found that paeonol not only significantly reduced the formation of foam cells *in vitro*, but also inhibited atherosclerotic plaque area in aortas from $ApoE^{-/-}$mice. The anti-atherosclerotic effect of paeonol may be attributed to the upregulation of ABCA1 and downregulation of CD36 via promotion of HO-1 expression.

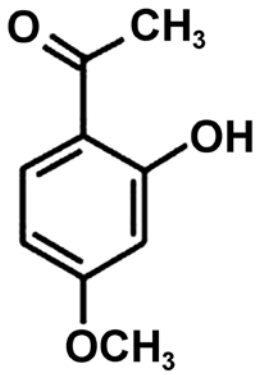

Figure 1. Chemical structure of paeonol.

Materials and methods

Reagents. Paeonol (purity, 98.0%) and cycloheximide (CHX) were purchased from Sigma (St. Louis, MO, USA). Goat anti-SR-A antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse anti-ABCA1 as well as anti-calpain, anti-calpastatin, anti-ABCG1, anti-CD36 and anti-SR-BI rabbit antibodies were from Abcam (Cambridge, MA, USA). Anti-HO-1, anti-c-Jun, anti-c-fos rabbit antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Assay kit for calpain activity was obtained from BioVision (Lyon, France). Scrambled and HO-1 small hairpin RNAs (shRNAs) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). ox-LDL was supplied by Guangzhou Yiyuan Biotechnology Co., Ltd. (Guangzhou, China). 3-Dodecanoyl-NBD cholesterol was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA).

Cell culture and transfection. RAW264.7 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA), and maintained in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum in a 37˚C incubator with 5% CO_2. An shRNA-containing plasmid that targeted HO-1 was designed and constructed by Shanghai GeneChem Co., Ltd. (Shanghai, China). The gene sequence of the shRNA was 5'-GCTGACAGAGGAACACAAAGA-3'. Cell transfections were performed with the SuperFect fragment (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions using scrambled or HO-1 shRNA in a 50-ml flask. Cells were incubated for 24 h after transfection and used for the indicated experiments (8).

Animals. Eight-week-old male $ApoE^{-/-}$ mice and C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME, USA), which were a generous gift from Dr Xiong-Zhong Ruan (Key Laboratory of Lipid and Glucose Metabolism, Chongqing, China), were housed in barrier facilities on a 12-h light / dark cycle. All experimental mice were allowed access to food and water *ad libitum*. Animal procedures were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) (Item no. 201303).

Animal experimental protocols. After being fed a high-fat diet (15.8% fat and 1.25% cholesterol) for 8 weeks, $ApoE^{-/-}$ mice were treated orally for 8 weeks with paeonol (150 mg/kg/day), simvastatin (5 mg/kg/day), or vehicle (20 ml/kg/day, 0.5% sodium carboxyl methyl cellulose) by gastric gavages (n=10, each group). The doses of paeonol and simvastatin used in this study is according to previous reports (22,23). C57BL/6J mice on a common diet as a control group (n=10) were treated with the same volume of vehicle only over the same treatment period. Eight weeks after treatment with paeonol or simvastatin (total diet-fed period was 16 weeks), mice were euthanized with CO_2, hearts and aortas were collected for Oil Red O staining and western blotting.

Atherosclerotic lesions at the level of the aortic valve were detected by Oil Red O staining (24) and atherosclerotic plaque area in the entire aorta was determined as described before (25).

Cell viability assay with MTT. Macrophages were seeded at density of $7.5x10^4$ cells/well in 96-well plates and the cell viability was determined by methylthiazolyl tetrazolium (MTT) assay. After the treatment with or without paeonol for 24 h, the culture supernatant was removed. The following procedures were performed as previously described (8).

Assessment of foam cell formation by Oil Red O staining. After washing 3x with PBS, cells were fixed in 4% paraformaldehyde for 20 min and then stained with 0.5% Oil Red O staining for 10 min to visualize cellular lipid accumulation. Hematoxylin was used as counter staining. The stained cells were photographed by light microscopy with x400 magnification. The density of lipid content was detected by alcohol extraction after Oil Red O staining. The absorbance at 540 nm was determined with a microplate reader.

Cholesterol efflux assay. We have previously applied NBD cholesterol to study the experiments of cholesterol efflux in macrophages (8). Macrophages were incubated with different concentration of paeonol for 24 h, followed by a further 6-h treatment of the equilibration of NBD cholesterol (1 μg/ml) in the presence of paeonol. NBD cholesterol-labeled cells were washed with phosphate-buffered saline and incubated in RPMI-1640 medium for 6 h. The fluorescence-labeled cholesterol released from the cells into the medium was measured

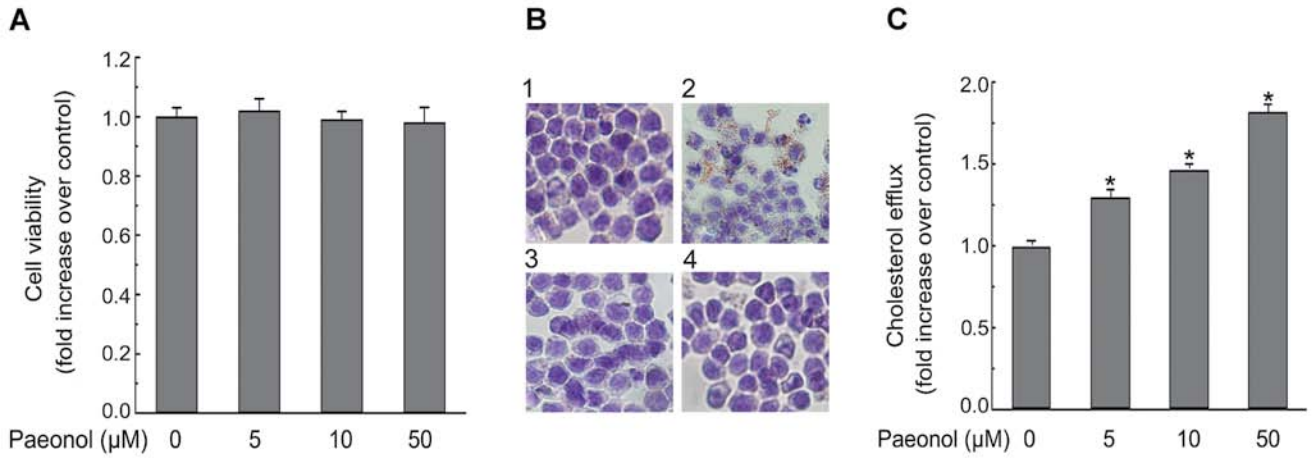

Figure 2. Paeonol at concentrations without cytotoxicity inhibits lipid accumulation and promotes cholesterol efflux in RAW264.7 macrophages. (A) Macrophages were treated with paeonol (0, 5, 10, 50 μM) for 24 h and cell viability was measured by methylthiazolyl tetrazolium (MTT) assay. (B) Macrophages were treated with oxidized low-density lipoprotein (ox-LDL) (50 μg/ml) in the presence or absence of paeonol (10 μM) for 24 h. 1, Control group; 2, the ox-LDL-treated group; 3, the ox-LDL plus paeonol group; 4, the paeonol-treated group. The cells with various treatments were fixed and then stained with Oil Red O. Magnification x400. (C) Cholesterol efflux assay was performed as described in the Materials and methods. The data are representative of three independent experiments (means ± SEM). *P<0.05 compared with control.

by use of a multilabel counter (PerkinElmer, Waltham, MA, USA) with 485 nm excitation and 535 nm emission.

Western blotting. Tissues or cells were harvested and protein extracts prepared as previously described (26). They were then subjected to western blotting using primary antibodies. The proteins were visualized and quantified using a chemiluminescence method (Pierce Biotechnology, Inc., Rockford, IL, USA) and Quantity One (Bio-Rad, Hercules, CA, USA) software program.

Immunoprecipitation. Cell lysates containing equal amounts of protein (1,000 mg) from macrophages treated with or without paeonol for 24 h were incubated with specific primary antibody overnight at 4˚C, and then with protein A/G-Sepharose for 2 h. Immune complexes were collected and eluted in lysis buffer. Eluted protein samples were then boiled in SDS-PAGE loading buffer for subsequent western blotting.

Quantitative real-time polymerase chain reaction (RT-qPCR). Total RNA was isolated by TRIzol reagent (Invitrogen Life Technologies). cDNA synthesis was performed using MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using a SYBR-Green PCR Master Mix kit [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China]. Primer sequences were as follows: SR-A forward, 5'-TGGTCCACCTGGTGCTCC-3' and reverse, 5'-ACCTCCAGGGAAGCCAATTT-3'; CD36 forward, 5'-CAGTTGGAGACCTGCTTATCC-3' and reverse, 5'-GCGTCCTGGGTTACATTTTC-3'; β-actin forward, 5'-TTGTCCCTGTATGCCTCTGG-3' and reverse, 5'-GAGGTCTTTACGGATGTCAACG-3'; ABCA1 forward, 5'-GGTTTGGAGATGGTTATACAATAGTTGT-3' and reverse, 5'-CCCGGAAACGCAAGTCC-3'; ABCG1 forward, 5'-TTCCCCTGGAGATGAGTGTC-3' and reverse, 5'-CAGTAGGCCACAGGGAACAT-3'; SR-BI forward, 5'-ACCCTAACCCAAAGGAGCAT-3' and reverse, 5'-CACAGCAACGGCAGAACTAC-3'. The reaction of RT-qPCR was performed under the following conditions: 3 min at 95˚C for 1 cycle, 10 sec at 95˚C, 30 sec at 60˚C for 39 cycles, 95˚C for 5 sec.

Measurement of calpain activity. Calpain activity was measured as previously described (3). Briefly, cellular lysates (100 mg) were mixed with reaction buffer and fluorogenic substrate Ac-LLY-AFC. The level of released AFC was measured >1 h at 37˚C by fluorometry using 400 nm excitation and 505 nm emission filters.

Statistical analysis. Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc test or unpaired Student's t-test. Continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test. Differences were considered statistically significant when P<0.05. All calculations were carried out with SPSS 15.0 version.

Results

Paeonol inhibits lipid accumulation and promotes cholesterol efflux in RAW264.7 macrophages. To investigate the toxic effect of paeonol treatment, cell viability was determined using an MTT assay. As shown in Fig. 2A, paeonol treatment for 24 h did not influence cell viability at the tested concentrations (5, 10, 50 μM). A similar result was reported previously (27). Therefore, a concentration range of 5-50 μM was chosen for subsequent experiments. Lipid accumulation, a hallmark of foam cell formation, was detected in cells treated with ox-LDL in the presence or absence of paeonol. Intracellular lipid accumulation in ox-LDL-treated macrophages was significantly declined when macrophages were co-incubated with paeonol and ox-LDL as revealed by Oil Red O staining (Fig. 2B). The data suggest that paeonol retarded ox-LDL uptake and foam cell formation in macrophages. Accordingly, paeonol treatment caused a

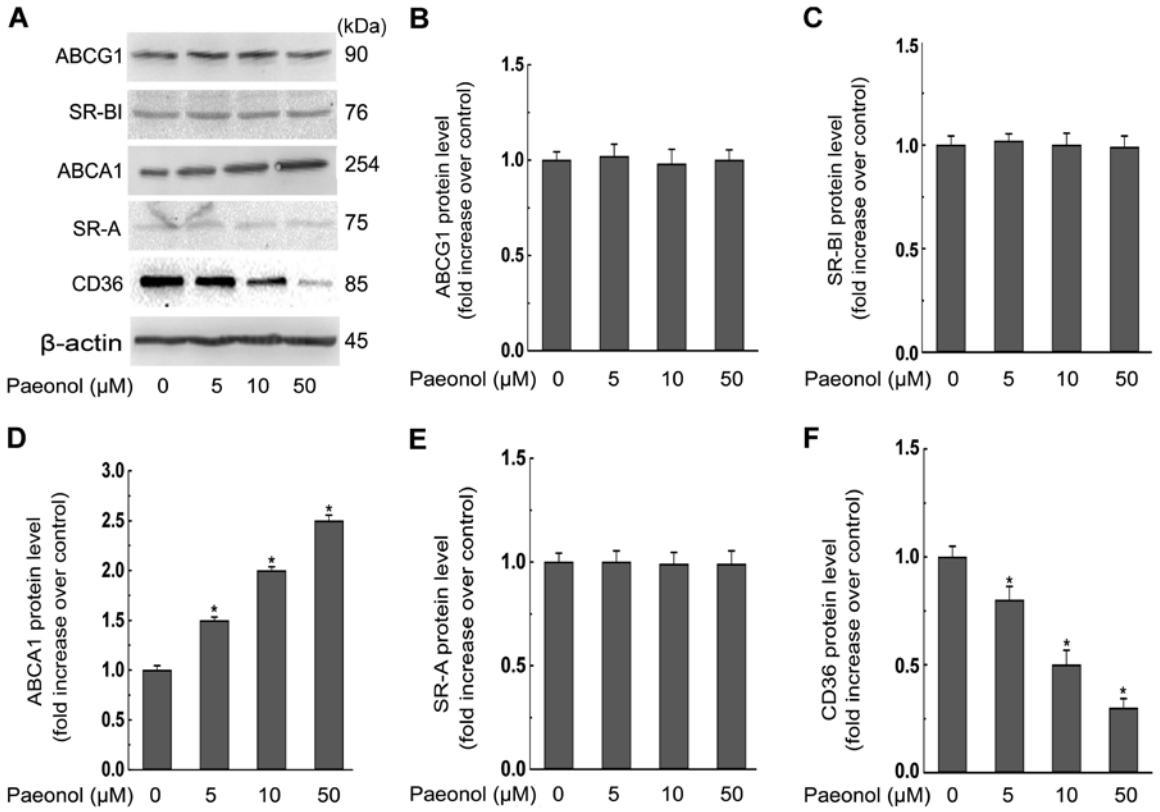

Figure 3. Paeonol decreases protein expression of the cluster of differentiation 36 (CD36), but increases protein expression of the ATP-binding cassette transporter A1 (ABCA1) in RAW264.7 macrophages. (A) Macrophages were treated with indicated concentrations (0, 5, 10, 50 μM) of paeonol for 24 h and the protein level of scavenger receptor class A (SR-A), CD36, SR-B type I (SR-BI), ABCA1, ABCG1, or β-actin was determined by western blotting. (B-F) The relative protein levels of CD36, SR-A, SR-BI, ABCA1, ABCG1 are presented as mean ± SEM of optical density from three separated experiments. *P<0.05 compared with control.

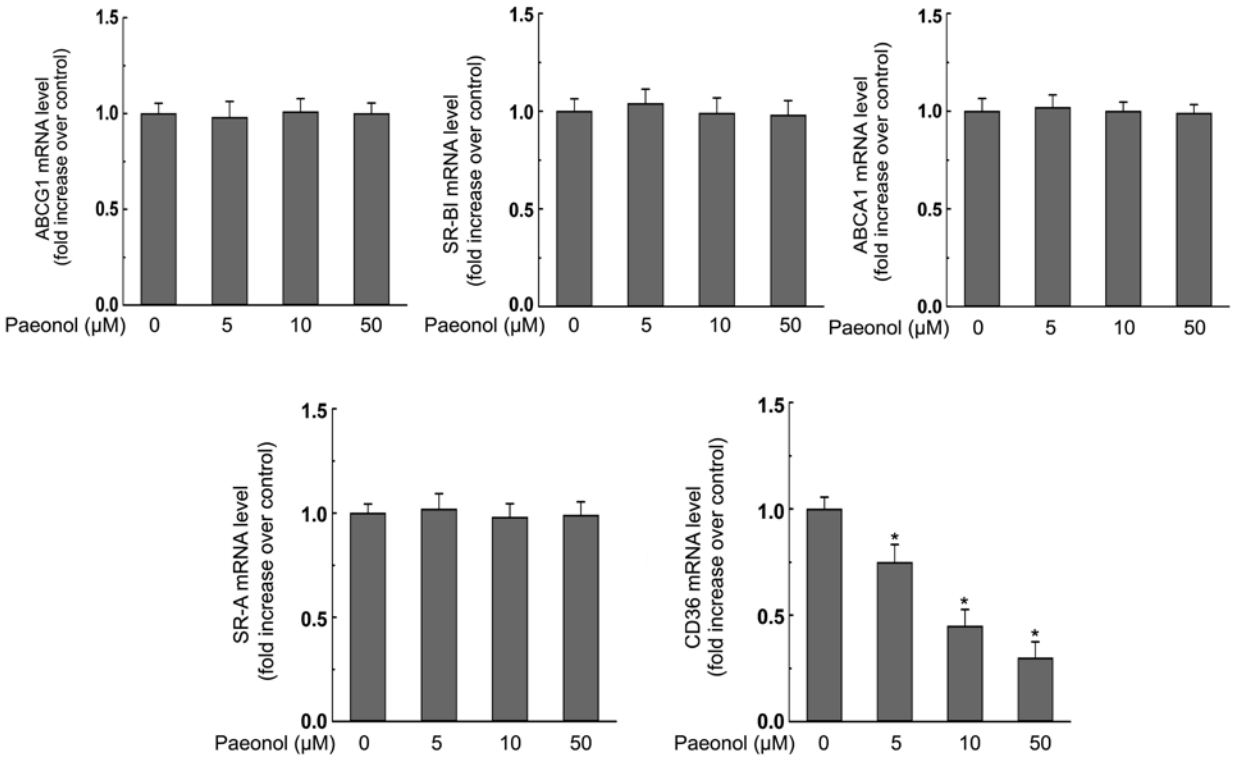

Figure 4. Paeonol decreases the mRNA expression of the cluster of differentiation 36 (CD36) in RAW264.7 macrophages. Macrophages were treated with paeonol (0, 5, 10, 50 μM) for 24 h. After treatment, total RNA was extracted and then subjected to quantitative real-time polymerase chain reaction (RT-qPCR) to detect the mRNA expression of scavenger receptor class A (SR-A), CD36, ATP-binding cassette transporter A1 (ABCA1), ABCG1, SR-B type I (SR-BI). The data are representative of three independent experiments (means ± SEM). *P<0.05 compared with control.

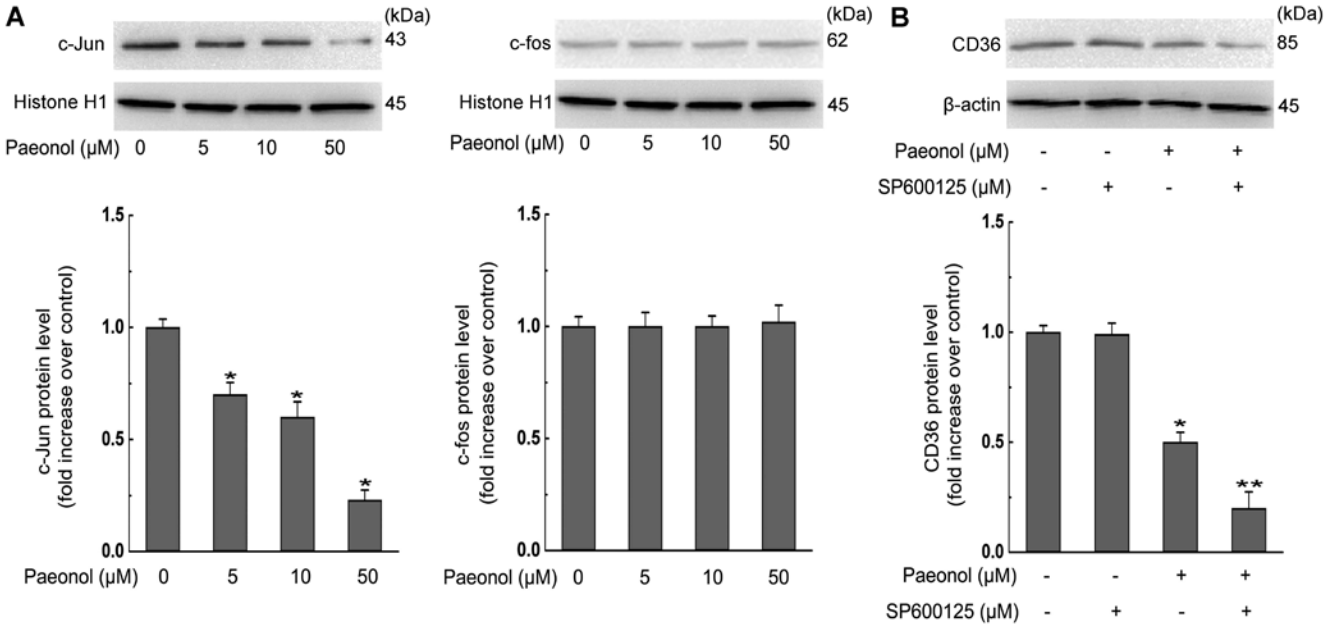

Figure 5. Nuclear translocation of c-Jun is involved in paeonol-mediated downregulation of the cluster of differentiation 36 (CD36) expression in RAW264.7 macrophages. (A) Macrophages were treated with paeonol (0, 5, 10, 50 μM) for 6 h, nuclear extracts were prepared and the protein level of c-fos, c-Jun was detected by western blotting. (B) Macrophages were pre-treated with SP600125 (10 μM) for 1 h, and then treated in the presence or absence of paeonol (10 μM) for 24 h. Protein expression of CD36 was determined by western blotting. The data are representative of three independent experiments (means ± SEM). *P<0.05 compared with control, **P<0.05 compared with paeonol-treated group.

dose-dependent increase of cholesterol efflux in macrophages as performed by using NBD-labeled cholesterol (Fig. 2C). This is likely to contribute to the protective effect by paeonol on macrophage foam cell formation.

Paeonol decreases the expression of CD36, but increases the expression of ABCA1 in RAW264.7 macrophages. We next explored the effect of paeonol on the expression of ABCA1, ABCG1, SR-BI, SR-A, CD36, which are reported to play the most critical roles during the lipid accumulation in macrophages (3). We found that paeonol treatment at various concentrations (0, 5, 10, 50 μM) for 24 h dose-dependently decreased both mRNA and protein expression of CD36 but had no effect on the expression of SR-A (Figs. 3 and 4). Additionally, the mRNA and protein expression of ABCG1 and SR-BI were not affected by paeonol either (Figs. 3 and 4). However, ABCA1 protein level in macrophages was promoted significantly in response to paeonol treatment (Fig. 3). Importantly, paeonol did not affect the mRNA expression of ABCA1 (Fig. 4).

c-Jun-AP-1 pathway is involved in the inhibition effect of paeonol on CD36 expression in RAW264.7 macrophages. c-Jun and c-fos [the subunits of activator protein-1 (AP-1)] have been reported to participate in CD36 and SR-A expression in macrophages (3,8). Therefore, we investigated the role of AP-1 in paeonol-invoked inhibition of CD36 expression. As shown in Fig. 5A, treatment of macrophages with paeonol caused a dose-dependent decrease in c-Jun nuclear level without affecting c-fos nuclear level. A c-Jun N-terminal kinase (JNK) inhibitor SP600125, which is a potent, cell-permeable selective and reversible inhibitor of JNK1, 2, and 3, augmented paeonol-induced decrease in CD36 expression (Fig. 5B). Collectively, these results show that the suppression of CD36 expression and subsequent alleviation of foam cell formation by paeonol are partly c-Jun-AP-1-dependent.

Decreased calpain activity is required for paeonol-invoked stabilization of ABCA1 protein in RAW264.7 macrophages. We further examined the molecular mechanisms involved in the effect of paeonol on the protein expression of ABCA1 by examining the protein stability of ABCA1 with paeonol treatment. In the presence of CHX (an inhibitor of *de novo* protein synthesis), the degradation rate of ABCA1 protein was dose-dependently inhibited by paeonol treatment (Fig. 6A and B). To define the possible mechanisms underlying the effect of paeonol on protein stability of ABCA1, we evaluated the activity of calpain, a protease involved in ABCA1 proteolysis, after the treatment of paeonol for 24 h in macrophages. We found that paeonol inhibited calpain activity in a dose-dependent manner (Fig. 6C). However, the expression of calpain and calpastatin (the endogenous inhibitor for calpain) were not altered by paeonol (Fig. 6D and E). Obviously, the declined calpain activity resulted from an increase in the protein interaction between calpain and calpastatin (Fig. 6F).

The suppression effect of paeonol on foam cell formation is regulated by HO-1. We explored the role of HO-1 in paeonol-induced inhibition of foam cell formation. The protein level of HO-1 in macrophages was dose-dependently increased in response to paeonol as shown in Fig. 7A. Moreover, transfection of the HO-1 shRNA at the concentration of 600 nM reversed the induction effect of paeonol on HO-1 protein expression in macrophages (Fig. 7B), whereas transfection with corresponding scrambled shRNA failed to do so. Additionally, HO-1 shRNA transfection decreased paeonol-invoked effects

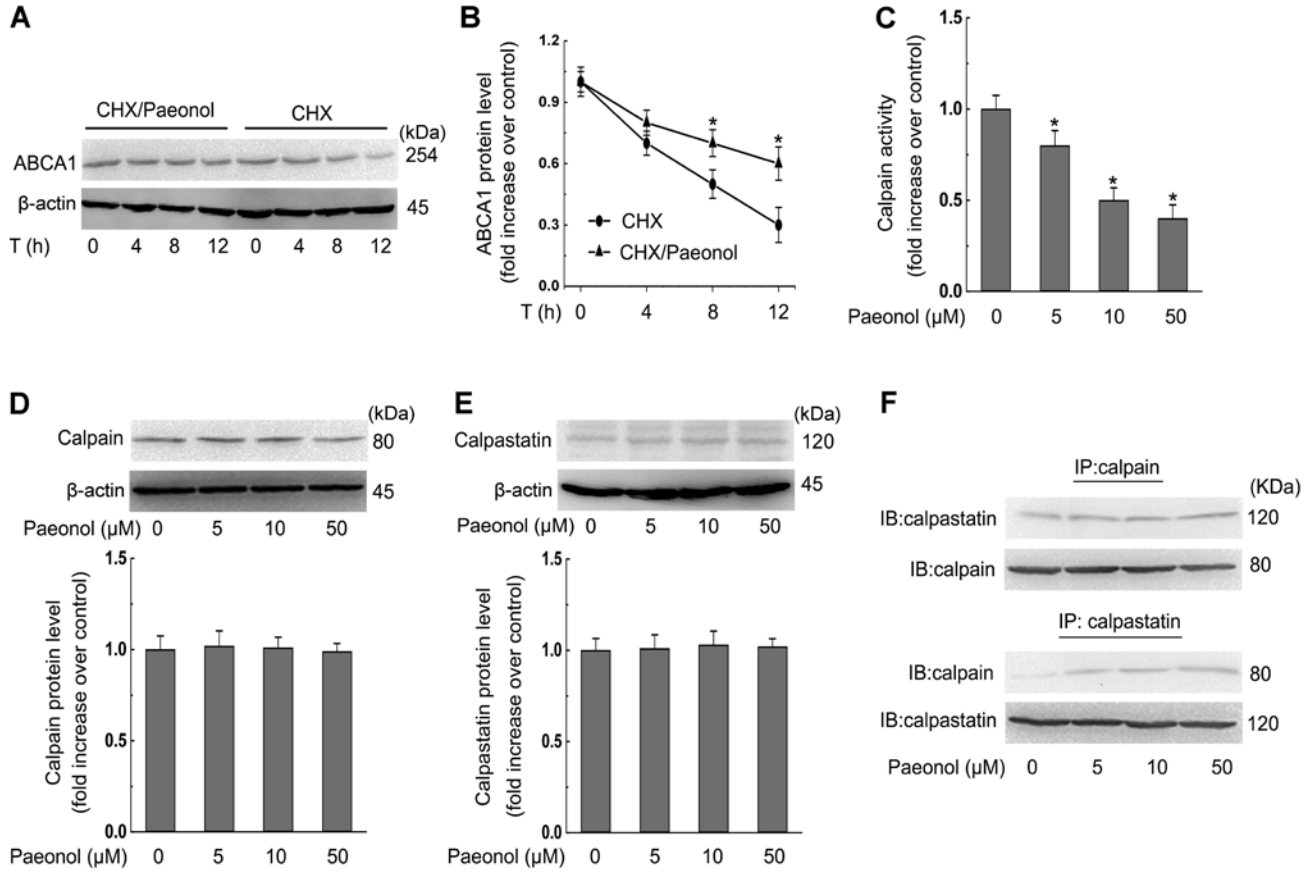

Figure 6. Paeonol enhances the stability of ATP-binding cassette transporter A1 (ABCA1) protein and decreases the calpain activity. (A and B) Macrophages were incubated with or without paeonol (10 μM) in the presence of cycloheximide (CHX) (2 μg/ml) for the indicated times. Cellular lysates were subjected to western blotting to determine the level of ABCA1 or β-actin. (C) Macrophages were treated with indicated concentrations of paeonol for 24 h and the calpain activity was determined. (D and E) Cellular lysates were subjected to western blotting to determine the expression of calpain, calpastatin, or β-actin. (F) Cellular lysates were immunoprecipitated (IP) with anti-calpain or anti-calpastatin antibody and then immunoprobed (IB) with anti-calpastatin or anti-calpain antibody. The data are representative of three independent experiments (means ± SEM). *P<0.05 compared with control.

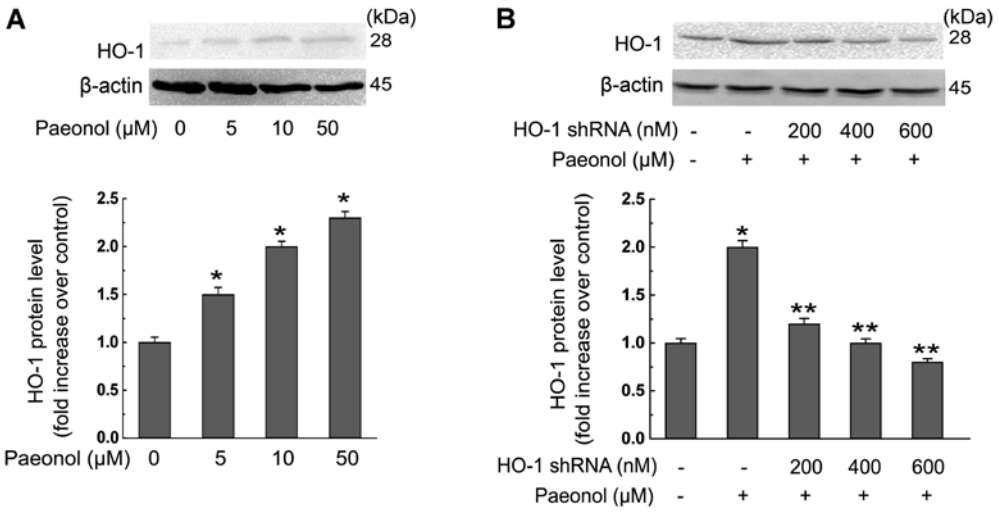

Figure 7. Paeonol-promoted haem oxygenase-1 (HO-1) protein expression was reversed by HO-1 small hairpin RNA (shRNA) in RAW264.7 macrophages. (A) Macrophages were incubated with various concentrations (0, 5, 10, 50 μM) of paeonol for 12 h. Western blotting was used to detect the protein expression of HO-1 after the treatment. (B) Macrophages were transfected with various concentrations of HO-1 shRNA (200, 400, 600 nM) for 24 h, followed by paeonol treatment (10 μM) for additional 12 h. Protein expression of HO-1 and β-actin was measured by western blotting. The data are representative of three independent experiments (means ± SEM). *P<0.05 compared with control, **P<0.05 compared with paeonol-treated group.

on the inhibition of c-Jun (Fig. 8A), and CD36 protein expression (Fig. 8B), promotion of ABCA1 protein expression (Fig. 8C), attenuation of calpain activity (Fig. 8D), and lipid accumulation (Fig. 8E) in macrophages, indicating the

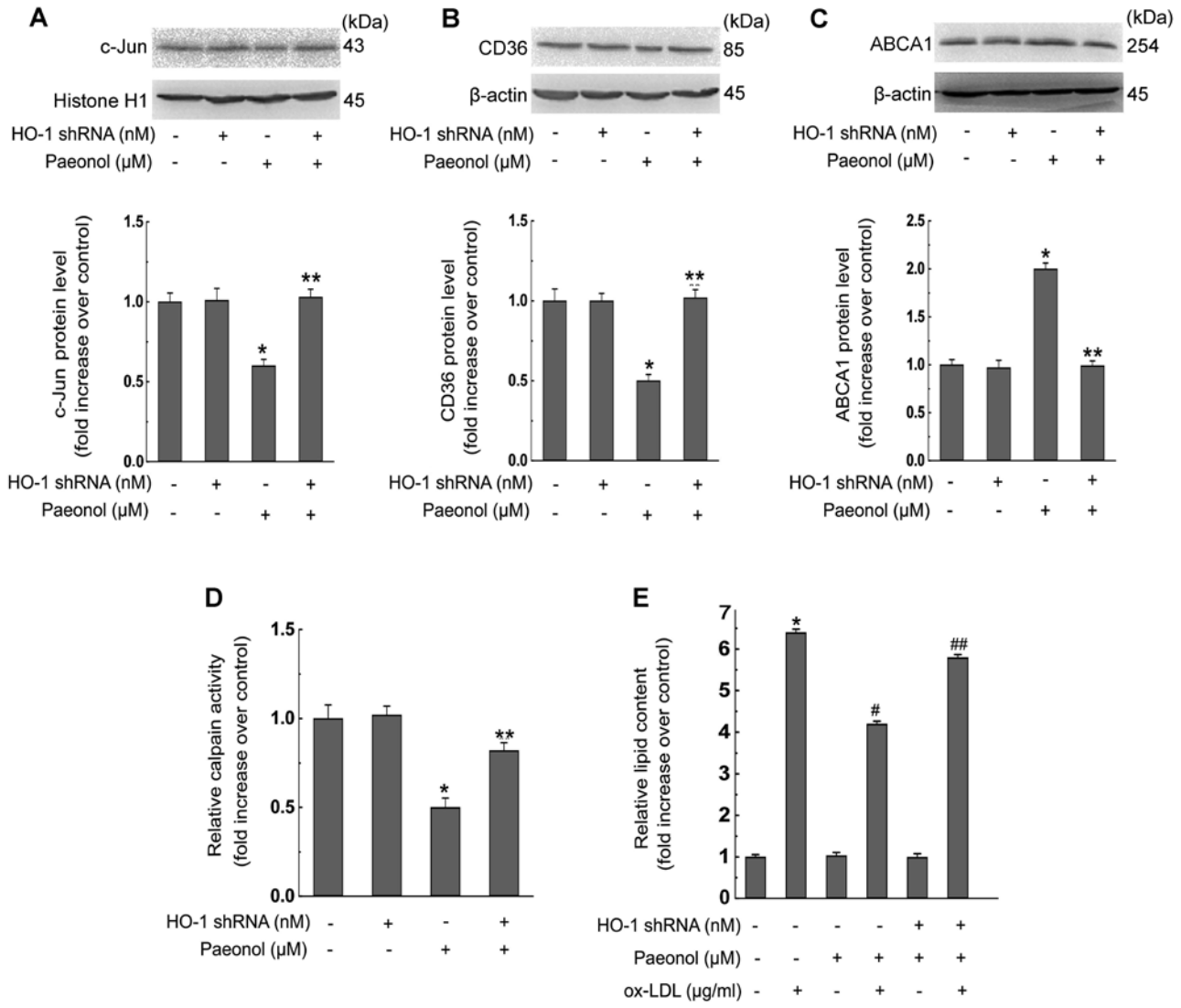

Figure 8. Paeonol-invoked athero-protective effects in macrophages are regulated by haem oxygenase-1 (HO-1). (A-C) Macrophages were pre-treated with HO-1 small hairpin RNA (shRNA) (600 nM) for 24 h, followed by paeonol for additional (A) 6 h or (B and C) 24 h. Protein level of c-Jun, β-actin, cluster of differentiation 36 (CD36) and ATP-binding cassette transporter A1 (ABCA1) was determined by western blotting. (D) Calpain activity was measured by using an enzymatic method. (E) Oxidized low-density lipoprotein (ox-LDL)-induced lipid accumulation was measured by alcohol extraction. The data are representative of three independent experiments (means ± SEM). *P<0.05 compared with control, **P<0.05 compared with paeonol-treated group, #P<0.05 compared with ox-LDL-treated group, ##P<0.05 compared with paeonol plus ox-LDL group.

crucial role of HO-1 in paeonol-mediated protection in foam cells.

Paeonol decreases atherosclerotic lesion formation in ApoE$^{-/-}$ mice. We further determined the anti-atherogenic function of paeonol *in vivo*. Morphological observations as shown in Fig. 9A and B, the lesion area in the aortic root was increased significantly in the ApoE$^{-/-}$ mice treated with vehicle compared with the control group. Treatment of 16-week-old ApoE$^{-/-}$ mice with paeonol (150 mg/kg) for 8 weeks significantly reduced atherosclerotic lesion formation compared to ApoE$^{-/-}$ mice treated with vehicle. However, no significant differences were found in lesion areas between paeonol-treated group and simvastatin-treated group. Similar results were obtained in atherosclerotic plaque formation as revealed by Oil Red O staining of whole aortas (Fig. 9C and D). Moreover, paeonol treatment increased ABCA1 protein expression in aortas, while decreased CD36 protein in aortas (Fig. 10). These results are consistent with the findings of *in vitro* experiments.

Discussion

It is well established that paeonol confers protection against atherosclerosis (16-20). Nevertheless, the effect and molecular mechanism by which paeonol mediates lipid accumulation in macrophage-derived foam cells remained to be resolved. We provide new insights into the molecular mechanisms involved in the anti-atherogenic property of paeonol in the macrophage-derived foam cell formation and in ApoE$^{-/-}$ mouse aortas. In RAW264.7 macrophages, paeonol treatment decreased ox-LDL-induced lipid accumulation by promoting ABCA1-dependent cholesterol efflux and attenuating CD36-dependent ox-LDL uptake. The increased ABCA1-regulated cholesterol efflux resulted from increased

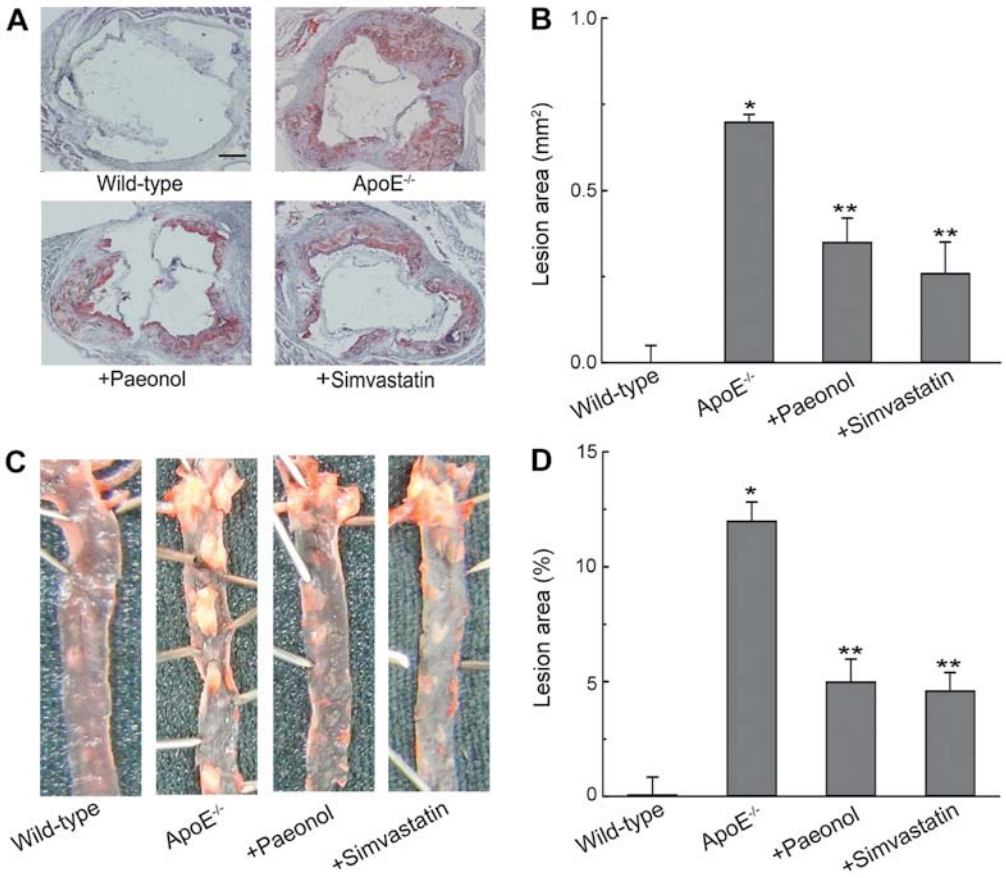

Figure 9. Paeonol inhibits atherosclerotic lesion formation in apolipoprotein E-deficient (ApoE$^{-/-}$) mice. (A) Representative Oil Red O stained aortic sections (x40 magnification; scale bar, 100 μm). (B) Quantification of Oil-red-O staining (n=10). (C) Typical morphology of aorta stained by Oil Red O. (D) Mean aortic lesion percentage in aortas (n=6). Wild-type, C57BL/6J mice; paeonol, paeonol at 150 mg/kg/day; simvastatin, simvastatin at 5 mg/kg/day. Quantitative analysis of plaque area was performed by two blinded observers using image analysis software (MetaMorph software; MDS Analytical Technologies, Inc., Sunnyvale, CA, USA). *P<0.05 compared with wild-type, **P<0.05 compared with ApoE$^{-/-}$.

protein stability of ABCA1. Our results strongly indicate that paeonol has a beneficial function in maintenance of lipid level during the transformation of foam cells in atherosclerosis and we delineated the underlying molecular mechanism.

SR-dependent ox-LDL internalization and RCT-mediated cholesterol efflux play a key role in the mediation of intracellular lipid level of foam cells (28). We may be the first to demonstrate that attenuation of intracellular lipid accumulation by paeonol in macrophage foam cells is possibly via decreased ox-LDL uptake and increased cholesterol efflux. In addition, paeonol significantly decreased both the protein and mRNA expression of CD36 without affecting SR-A expression. SR-A and CD36 are two major types of SRs involved in the uptake of ox-LDL in macrophages (29). Genetic inactivation of CD36 reduced ox-LDL uptake *in vitro* and atherosclerotic lesions in mice (30). Extensive research also indicates that CD36 expression can be decreased by anti-atherogenic antioxidants, which reveals its key role in the pathogenesis of atherogenesis (31,32). In the present study, we demonstrate that the suppressive effect of paeonol on ox-LDL uptaking was via downregulation of CD36 expression, which could be caused by decreased nuclear translocation of c-Jun (a subunit of AP-1). Indeed, pharmacological inhibition of AP-1 pathway augmented the effect of paeonol on CD36 expression. Our findings show that inactivation of the c-Jun-AP-1 pathway is required for the athero-protective action of paeonol in macrophages. In view of the function of CD36, the attenuation of CD36 expression by paeonol may contribute to the reduction of ox-LDL internalization and subsequent inhibition of foam cell formation.

In addition to its inhibitory effect on CD36 expression, paeonol promoted cholesterol efflux by increasing ABCA1 protein expression without altering ABCA1 mRNA level. The increase of ABCA1 protein expression induced by paeonol likely results from increased stability of ABCA1 protein. ABCA1 is the most important RCT responsible for cholesterol efflux from macrophage foam cells (28). Defects in ABCA1-mediated reverse cholesterol transport result in increased intracellular cholesterol accumulation, and ABCA1 plays a critical role in protecting against atherosclerosis in both humans and animals (33). Moreover, the expression of ABCA1 is known to be upregulated by various antioxidants with anti-atherogenic properties (34,35). In view of ABCA1 function, the increased expression of ABCA1 by paeonol observed in this study is also likely to facilitate the cholesterol efflux and subsequent suppression of foam cell formation. Importantly, our findings indicate that the induced expression of ABCA1 by paeonol is accompanied by decreased calpain activity. We further show that the reduced calpain activity may be due to the increased protein interaction between calpain and calpastatin as revealed by immunoprecipitation. The critical role of calpain in the stabilization of ABCA1 protein

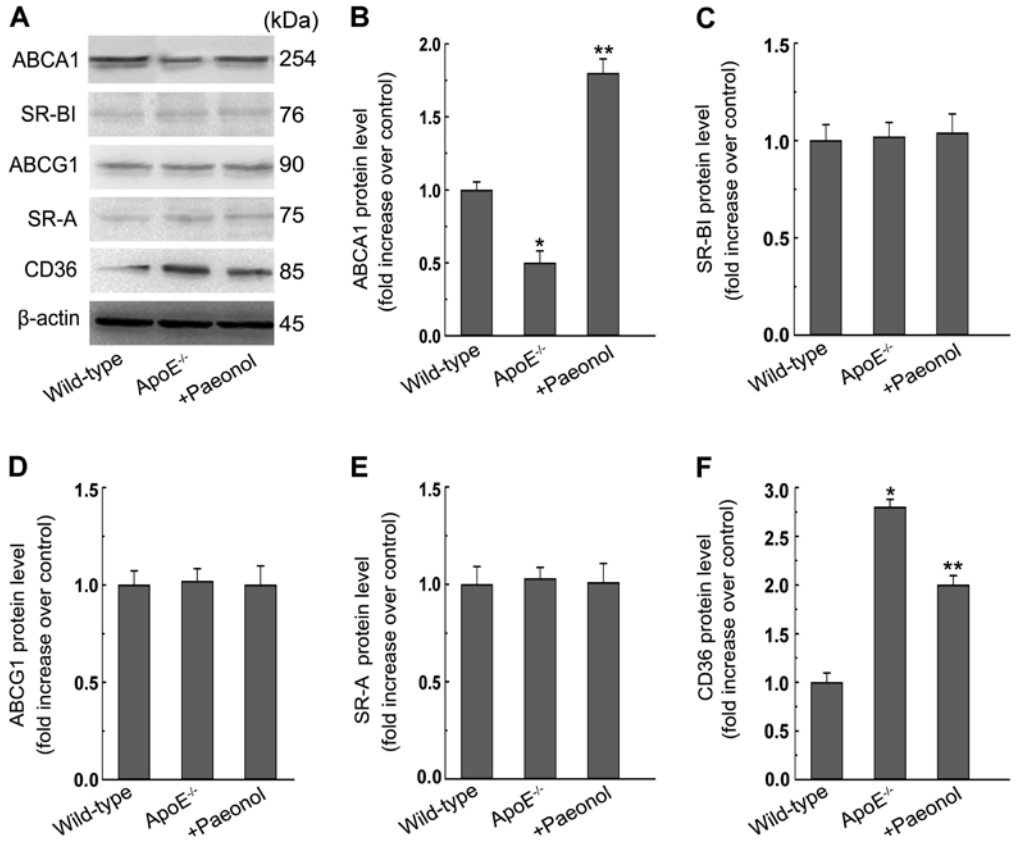

Figure 10. Paeonol promotes ATP-binding cassette transporter A1 (ABCA1) protein expression, but decreases the protein expression of the cluster of differentiation 36 (CD36) in aortas of apolipoprotein E-deficient ($ApoE^{-/-}$) mice. (A) After the treatment of wild-type or $ApoE^{-/-}$ mice as described in the Materials and methods section, aortas were collected and subjected to western blotting to detect the protein expression of ABCA1, ABCG1, SR-B type I (SR-BI), scavenger receptor class A (SR-A), CD36. One representative blot is shown. (B-F) The relative protein levels of CD36, SR-A, SR-BI, ABCA1, ABCG1 are presented as mean ± SEM of optical density from three separated experiments. $^{*}P<0.05$ compared with wild-type, $^{**}P<0.05$ compared with $ApoE^{-/-}$. Wild-type, C57BL/6J mice; paeonol, paeonol at 150 mg/kg/day.

is well established (36,37). Furthermore, wogonin increases the protein stability of ABCA1 via PP2B-mediated dephosphorylation (38). Whether PP2B-mediated dephosphorylation is involved in paeonol-invoked increase in ABCA1 protein stability remains to be further examined.

Ample studies reported that HO-1 is correlated with the suppression of atherosclerotic damage (39,40). Indeed, HO-1 overexpression by pharmacological inducers or viral gene transfer inhibits atherogenesis in hypercholesterolemic animal models (39,41). On the other hand, our previous study shows that gene knockdown of HO-1 reversed kaempferol-invoked protection against foam cell formation (8). However, whether HO-1 is involved in the athero-protective action of paeonol remains unknown. In this study, we found that paeonol profoundly elicited HO-1 protein expression in macrophages. Additionally, our findings indicated that gene knockdown of HO-1 prevented paeonol effects on the inhibition of c-Jun/AP-1 nuclear translocation, increase of ABCA1 expression, decrease of CD36 expression, attenuation of calpain activity, and lipid accumulation in macrophages. Collectively, these data strongly suggest the essential role of HO-1 in the paeonol-mediated suppression of foam cell formation. Moreover, the metabolites of HO-1, biliverdin and carbon monoxide, are known to provide protection in cardiovascular diseases (42). Nevertheless, whether these metabolites are involved in the anti-atherogenic effect of paeonol on the formation of foam cells remains to be examined in the future.

Our findings of paeonol-invoked inhibition of foam cell formation are not limited to the cell culture system. Our *in vivo* experiments indicated that the positive drug simvastatin decreases atherosclerotic lesion formation, which is consistent with a previous study (22). This result suggests that the atherosclerotic model was established successfully. Moreover, paeonol retarded atherosclerotic progression in $ApoE^{-/-}$ mice. In fact, studies have reported that paeonol displayed several beneficial effects on atherosclerosis such as inhibition of monocyte adhesion to vascular endothelial cells (43), inhibiting inflammatory response (16) and downregulating platelet aggregation (15). Here, we further found increased protein levels of ABCA1 and decreased protein expression of CD36 in paeonol-treated $apoE^{-/-}$ mice. Hence, these therapeutic effects seem to be widespread, but the target cells for paeonol cannot be identified under this hyperlipidemic situation. Accordingly, paeonol may have beneficial effect in various organs via dissimilar mechanisms.

In conclusion, our data show that HO-1 plays a crucial role in the anti-atherogenic effect of paeonol on the formation of foam cells, which reduces intracellular lipid accumulation in foam cells via downregulating CD36 and post-transcriptionally upregulating ABCA1 expression. We provide new insights for better understanding the molecular mechanisms involved in paeonol-invoked inhibition of foam cells formation in atherosclerosis. Paeonol may exert anti-atherosclerotic effect by reducing serum lipid. Whether paeonol could inhibit serum

lipid and the involved mechanisms in apoE$^{-/-}$ mice need to be investigated in the future.

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