Original Paper

Leonurine Prevents Atherosclerosis Via Promoting the Expression of ABCA1 and ABCG1 in a Ppar γ /Lxr α Signaling Pathway-Dependent Manner

Ting Jiang^a Kun Ren^b Qian Chen^c Heng Li^c Rong Yao^c Hu Hu^c
Yun-Cheng Lv^d Guo-Jun Zhao^a

^aDepartment of Histology and Embryology, Guilin Medical University, Guilin, ^bInstitute of Cardiovascular Research, Key Laboratory for Atherosclerosis of Hunan Province, University of South China, Hengyang, ^cUndergraduate Student in Department of Clinical Medicine, Guilin Medical University, Guilin, ^dLaboratory of Clinical Anatomy, University of South China, Hengyang, China

Key Words

Leonurine • ATP-binding cassette transporter A1/G1 • Peroxisome proliferator-activated receptor γ • Liver X receptor • Cholesterol efflux • Atherosclerosis

Abstract

Background/Aims: Previous studies have demonstrated that leonurine, a unique alkaloid compound of *Herba leonuri*, can exert anti-oxidative and anti-inflammatory effects on the development of atherosclerosis (AS). This study was designed to investigate the effects of leonurine on cholesterol efflux from THP-1 macrophage-derived foam cells and development of atherosclerotic lesions in apoE^{-/-} mice, and further determine the potential mechanisms.

Methods: Human THP-1 cells were fully differentiated into foam cells by the pre-treatment with phorbol-12-myristate-13-acetate (PMA) and oxidized density lipoproteins (ox-LDL). After cells were incubated with various concentrations of leonurine, Oil Red O staining and high-performance liquid chromatography (HPLC) assays were utilized to detect cellular lipid accumulation and cholesterol content, respectively. Cellular cholesterol efflux was determined by liquid scintillation counting. The mRNA and protein levels of ATP-binding cassette transporter A1/G1 (ABCA1/G1), peroxisome proliferator-activated receptor γ (PPAR γ) and liver X receptor α (LXR α) in foam cells were assessed using real-time quantitative PCR (RT-qPCR) and western blot analyses, respectively. Plasma triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) levels in apoE^{-/-} mice were evaluated using enzymatic methods. The atherosclerotic lesion sizes and collagen contents in aortic roots were determined by Oil Red O and Masson's trichrome staining, respectively. **Results:** Oil Red O staining and liquid scintillation counting assays showed that leonurine significantly inhibited lipid accumulation and promoted ³H-cholesterol efflux in human THP-1 macrophage-derived foam cells in a concentration-dependent manner.

T. Jiang and K. Ren contributed equally to this work.

Guo-Jun Zhao, Ph.D.,
Yun-Cheng Lv, Ph.D.,

Department of Histology and Embryology, Guilin Medical University, Guilin, (China)
Laboratory of Clinical Anatomy, University of South China, Hengyang, (China)
Tel. 86-773-3680365, E-Mail zzhcsu@163.com/anthony0723@163.com

Besides, both the mRNA and protein levels of ABCA1/G1, PPAR γ and LXR α were enhanced by leonurine, which were attenuated by LXR α siRNA or PPAR γ siRNA transfection. Finally, leonurine improved plasma lipid profile, decreased atherosclerotic lesion sizes, increased collagen contents and amplified PPAR γ , LXR α and ABCA1/G1 expressions in aortic roots of apoE $^{-/-}$ mice. **Conclusions:** Leonurine can promote cholesterol efflux and alleviate cellular lipid accumulation by magnifying the expression of ABCA1/G1 in a PPAR γ /LXR α signaling pathway-dependent manner in human THP-1 macrophage-derived foam cells and abate atherogenesis in apoE $^{-/-}$ mice, which may offer a promising therapeutic intervention of leonurine in protecting against AS.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

Cardiovascular disease (CVD) is one of the leading causes of deaths around the world, the fundamental pathological basis of which is atherosclerosis (AS) [1-3]. The accumulation and deposition of macrophage-derived foam cells in vessel walls are the major feature in the progression of AS [4-6]. It has been well established that ATP-binding cassette transporter A1/G1 (ABCA1/G1) play a critical role in promoting cellular cholesterol efflux and regulating lipid metabolism [7]. Studies using ABCA1 $^{-/-}$ or ABCG1 $^{-/-}$ cells as well as animals have shown that down-regulation of ABCA1 or ABCG1 expression decreases cellular cholesterol efflux and increases lipid accumulation [8-10]. ABCA1 and ABCG1 are rate-limiting factors of high-density lipoprotein (HDL) assembly and the expression of ABCA1/G1 can be modulated by a large network of transcriptional and post-transcriptional factors [11].

Herba leonuri (HL) is a traditional Chinese herbal medicine, which has been widely used for the therapy of many disorders for many years, including gynecological disorders, menstrual disorders, and dysmenorrhea [12, 13]. Recently, it has been identified that leonurine, a natural alkaloid extract of HL, can attenuate atherogenesis through its anti-oxidative and anti-inflammatory properties, thus playing beneficial roles in CVD [14-16]. Zhang et al. [17] found that in hypercholesterolemic rabbits, leonurine can abate atherogenic process dose-dependently through the diminution of macrophage infiltration and smooth muscle cell (SMC) migration as well as the reduction of the expression of some cytokines, adhesion molecules, and chemokines. Liu's studies suggested that in human umbilical vein endothelial cells (HUVECs), leonurine can inhibit tumor necrosis factor- α (TNF- α)-induced HUVEC-monocyte interaction and decrease the levels of vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), cyclooxygenase-2 (COX-2), and monocyte chemotactic protein 1 (MCP-1) via repressing reactive oxygen species (ROS)-activated p38 mitogen-activated protein kinase (MAPK)/nuclear transcription factor (NF- κ B) pathway [18]. Another study [15] established that leonurine can prevent the H₂O₂-induced apoptosis of H9c2 cells by activating c-Jun N-terminal kinase 1/2 (JNK1/2) and improving mitochondrial dysfunction. However, whether leonurine is associated with cellular lipid accumulation and cholesterol metabolism is still unknown. Given that ABCA1 expression can be modulated by some pro-inflammatory factors and naturally occurring triterpenoids via the NF- κ B signaling pathway [19, 20], it is possible that leonurine regulates the ABCA1/G1 levels. Our results demonstrated that leonurine can enhance the expression of ABCA1/G1 and ABCA1/G1-mediated cholesterol efflux in THP-1 macrophage-derived foam cells in a peroxisome proliferator-activated receptor γ (PPAR γ)/liver X receptor α (LXR α) pathway-dependent fashion, likely contributing to its alleviation of atherogenesis in apoE $^{-/-}$ mice.

Materials and Methods

Cell culture and treatment

Human THP-1 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and cultured in RPMI-1640 medium containing 0.1% nonessential amino acids,

penicillin/streptomycin (100U/mL) and 20% fetal bovine serum (FBS) under standard culture conditions (5% CO₂ and 37 °C). Prior to their use in experiments, THP-1 cells were pre-treated with phorbol-12-myristate-13-acetate (PMA, 160 nmol/L; Sigma) for 24 h. Then, the medium was replaced with serum-free medium supplemented with ox-LDL (50 µg/ml) for another 48 h to fully differentiate THP-1 into macrophages.

Western Blot Analysis

Cells or murine tissues were collected for protein extraction and detection of ABCA1, ABCG1, PPAR γ and LXR α , as described previously [21]. The concentration of proteins was measured using a BCA protein assay kit (CWBIO, Peking, China). Proteins (20 µg per lane) were loaded on a 8% SDS-polyacrylamide electrophoresis gel (SDS-PAGE, Solarbio Co, Peking, China), electrophoresed at 100 V for 2 h in gel running buffer for separation, and subsequently transferred to 0.45 µm polyvinylidene fluoride membranes (PVDF, Merck Millipore, Darmstadt, Germany). The efficiency of protein transfer was assessed by Li Chunhong S staining (CWBIO, Peking, China). Thereafter, membranes were blocked using 5% fat-free milk dissolved in Tris-buffered saline with Tween-20 (TBS-T) for 4 h at room temperature and then incubated with primary antibodies against ABCA1 (1:1, 000, ab7360, Abcam, Cambridge, UK), ABCG1 (1:1, 000, ab52617, Abcam, Cambridge, UK), PPAR γ (1:1, 000, ab178860, Abcam, Cambridge, UK), LXR α (1:1, 000, ab176323, Abcam, Cambridge, UK) and β -actin (1:1, 000, ab8227, Abcam, Cambridge, UK) at 4°C with gentle shaking overnight. After a series of rinses, membranes were further incubated with a peroxidase-conjugated secondary antibody (1: 5, 000, B8895, Sigma-Aldrich) in TBS-T for 2 h at room temperature. Finally, proteins were visualized using a chemiluminescence method (enhanced chemiluminescence Plus Western Blotting Detection System; Amersham Biosciences, Foster City, CA) and the protein levels were evaluated semiquantitatively using Quantity One software.

RNA Extraction and Real-time PCR (RT-PCR)

RT-PCR analysis was performed as previously described [22, 23]. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and cDNA fragments (2 µg) were obtained by reverse transcription of RNA (1 µg) using a TaqMan Reverse Transcription Reagents Kit (Applied Biosystems). PCR analysis was conducted on a Roche Light Cycler Run 5.32 Real-Time PCR System with SYBR Green detection chemistry. The primers used were as follows: ABCA1 sense, 5'-TGTCTCGTATTGTCTGCGG-3' and anti-sense, 5'-GGGCTTTGTAGTTGTTGTCCT-3'; ABCG1 sense, 5'-GGTGGTCTCGCTGATGAAAG-3' and anti-sense, 5'-CTGCTGGGTTGTGGTAGGTT-3'; LXR α sense, 5'-ACACCTACATGCGTCGCAAG-3' and anti-sense, 5'-GACGAGCTTCTCGATCATGCC-3'; PPAR γ sense, 5'-TGGAATTAGATGACAGCGACTTGG-3' and anti-sense, 5'-CTGGAGCAGCTTGGCAAACA-3'; β -actin sense, 5'-ATCGTGCGTGACATTAAGGAGAAG-3' and anti-sense, 5'-AGGAAGGAAGGCTGGAAGAGTG-3'. All RT-PCR products were assessed by melt curve analyses, in which DNA duplexes were identified. Measurements were quantified using the $\Delta\Delta$ Ct method and β -actin expression was used as the internal control.

Cholesterol Efflux Assays

THP-1 cells (1×10⁶ cells/well) were cultured and differentiated as indicated above, and radiolabeled with 5 µCi/ml ³H-cholesterol (PerkinElmer, America). Then, the media were supplemented with different concentrations of leonurine for another 24 h. After washed twice with phosphate-buffered saline (PBS), cells were cultured in RPMI 1640 supplemented with 0.2% (w/v) BSA and human apoA-I (10 µg/ml, Sigma) or HDL (50 µg/ml, Sigma), as lipid acceptors. 6 h later, liquid scintillation counting was used to measure the ³H-cholesterol level in cells and medium, respectively. The percent efflux was quantified according to the following equation: [total media counts/(total media counts + total cellular counts)]×100% [24, 25].

Oil Red O staining

Oil Red O staining of THP-1 macrophage-derived foam cells was conducted as described previously [26]. Briefly, after human THP-1 cells were seeded into 6-well plates (1.0×10⁶ cells/well) and fully differentiated, cells were washed with PBS for several times and fixed in 4% paraformaldehyde (dissolved in PBS) for 10 min. Thereafter, macrophages were rinsed with 60% isopropanol and stained with 0.3% Oil Red O (dissolved in 60% isopropanol, Yiyuan Biotechnologies, Guangzhou, China) for 10 min and then rinsed with 60% isopropanol again. Then, Gill III hematoxylin (Sigma) was used to counterstain the cells for 5 min.

Following a series of rinses with double-distilled water, photographs of stained macrophages were obtained using a microscope at 40× magnification.

High-Performance Liquid Chromatography (HPLC) Assay

The protocols of HPLC assay were as follows: After cells were rinsed with PBS for several times, approximately 1 ml of 0.5% NaCl was added to 100–200 µg cellular proteins per ml. Then, an ultrasonic processor was used to sonicate the cells for 3 min. The concentration of proteins in cell solution was determined using a BCA kit. An equivalent bulk of freshly prepared cold KOH (-20 °C, dissolved in 150 g/L ethanol) was supplemented. Cell lysates were vortexed repeatedly until it was clear. An equivalent volume of isopropanol: hexane 2:3 (v/v) was then added. After the mixture was vortexed for 3 min, it was centrifuged at 800 rpm at 15°C for 7 min. The extraction procedure was repeated for three times. Then, approximately 0.1 ml of aliquot cell solution (containing 5~20 µg protein) was utilized to detect total cholesterol (TC) and another aliquot for free cholesterol (FC) measurement. Isopropanol was used to dissolve FC (1 mg cholesterol/ml), which was then kept at -20°C as a stock solution. By diluting the cholesterol stock solution, cholesterol standard calibration solution (0~40 µg cholesterol/ml) was prepared.

Subsequently, 10 µl reaction mixture (including 5% NaCl, 10 mM dithiothreitol, 500 mM Tris-HCl (pH 7.4), and 500 mM MgCl₂) was added to 0.1 ml of each sample (cell solutions, or cholesterol standard calibration solutions). Each tube was supplemented with 0.4 unit of cholesterol oxidase in 10 µl 0.5% NaCl for FC determination, or 0.4 unit of cholesterol oxidase and 0.4 unit of cholesterol esterase for TC assessment. The reaction process was performed at 37°C for 30 min and stopped by adding 100 µl of ethanol: methanol (1:1) to each tube. To allow protein precipitation, each tube was kept in cold environment for 20 min, and then centrifuged at 15°C at 1500 rpm for 10 min. Then, the supernatant (10 µl) was transferred onto a System Chromatographer (PerkinElmer Inc.), including a pump, a PerkinElmer series 200 UV/vis detector, a PerkinElmer series 600 LINK, a PerkinElmer series 200 vacuum degasser, and a Discovery C-18 HPLC column (Supercool Inc.). The elution of the column was performed using isopropanol: n-heptane: acetonitrile (35:13:52) at a flow rate of 1 ml/min for 10 min. Data were assessed using TotalChrom software from PerkinElmer with absorbance at 216 nm [14].

Small interfering RNA

Human THP-1 macrophages (1×10⁶ cells/well) were transfected with small-interfering RNA (siRNA) specific for LXRα (Santa Cruz Biotechnology, sense, 5'-GGCUGCAAGUGGAAUUCAUT T-3'; anti-sense, 5'-AUGAAUCCACUUGCAGCCTT-3' and PPARγ (Santa Cruz Biotechnology, sense, 5'-GGAUGCAAGGGUUCUUCCTT-3'; anti-sense, 5'-GGAAGAAACCCUUGCAUCCTT-3') using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Then, western blot analysis was conducted to evaluate the transfection efficiency.

Mice and Treatments

ApoE^{-/-} mice (male, eight-week old) were purchased from Laboratory Animal Center of Peking University, China. After fed a chow diet for 2 weeks, apoE^{-/-} mice were randomly divided into several groups (n=15/group). Mice in the leonurine group were intragastrically administered with leonurine (10 mg/kg/d) every day and continued for 8 weeks. The control group was fed with an equal volume of PBS. At week 16, mice were euthanized, followed by collecting the blood and tissue samples for further analyses.

The investigation strictly obeyed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised in 1996) and Care and Use guidelines for experimental animals of University of South China. The investigation procedure was approved by the Animal Ethics Committee of University of South China. To minimize sufferings, sodium pentobarbital anesthesia was performed prior to all surgeries.

Lipid Analyses

Mice were fasted overnight and sacrificed. Then, blood samples were obtained from the retro-orbital plexus. Plasma levels of TC, TG, HDL-C and LDL-C were measured by enzymatic methods using commercial test kit (Shanghai Rongsheng Biotech Inc. Shanghai, China).

Assessment of Aortic Lesions

Hearts and the proximal aortas were dissected and fixed in formalin. Under and parallel to the leaflet, hearts were cut directly, and the upward section was kept in OCT medium and stored at 4°C. Then, eight μm thick segments were obtained through the cleavage of aortic sinus. Twenty or more segments from each mouse were stained with Oil Red O and Masson's trichrome (MT) (Senbeibio, China) to identify lesion areas and collagen contents, respectively. Atherosclerotic lesions and collagen contents were determined using IMAGEPRO PLUS (Media Cybernetics, Silver Spring, MD). Values were presented as lesion size $\pm$ SEM [14].

Statistical Analysis

Data were obtained from at least three independent experiments and expressed as means $\pm$ standard deviation (SD). The results were analyzed by the Student's t-test using the SPSS 13.0 software. Statistical significance was taken as $p < 0.05$.

Results

Effects of leonurine on lipid accumulation and cholesterol efflux in human THP-1 macrophages

The development of AS is characterized by the deposition of foam cells within vessel walls [27]. Firstly, we evaluated the effects of leonurine on lipid accumulation, contents and cholesterol efflux from THP-1 macrophage-derived foam cells using Oil Red O staining, HPLC and liquid scintillation counting assays, respectively. After human THP-1 cells were fully differentiated, cells were incubated with different concentrations of leonurine (0, 5, 10, 20, 40, 80 μM) for 24 h. Results showed that treatment of cells with leonurine led to diminution in lipid accumulation (Fig. 1), cellular cholesterol content, including TC, FC and cholesteryl ester (CE) (Table 1), and increase in apoA-I- or HDL-mediated cholesterol efflux (Fig. 2), indicating that leonurine can prevent foam cell formation in a concentration-dependent manner.

Fig. 1. Effects of leonurine on lipid accumulation in THP-1 macrophage-derived foam cells. After THP-1 cells were fully differentiated, cells were treated with different concentrations of leonurine (0, 5, 10, 20, 40, 80 μM) for 24h, followed by Oil Red O staining.

Table 1. Effects of leonurine on cholesterol content in THP-1 macrophage-derived foam cells at different concentrations. After THP-1 cells were fully differentiated, cells were treated with different concentrations of leonurine (0, 5, 10, 20, 40, 80 μM) for 24 h. Then, HPLC analysis was performed to determine cellular TC, FC and CE levels. All results were obtained from three independent experiments, each performed in triplicate. Data were expressed as means $\pm$ SD. * $P < 0.05$ vs 0 μM group. TC, total cholesterol; FC, free cholesterol; CE, cholesteryl ester; * compared with control group, $p < 0.05$

Leonurine (μM)	0	5	10	20	40	80
TC (mg/g)	788 $\pm$ 17	677 $\pm$ 25*	611 $\pm$ 11*	523 $\pm$ 25*	455 $\pm$ 12*	275 $\pm$ 25*
FC (mg/g)	304 $\pm$ 19	280 $\pm$ 20*	241 $\pm$ 22*	217 $\pm$ 18*	174 $\pm$ 15*	112 $\pm$ 16*
CE (mg/g)	484 $\pm$ 24	404 $\pm$ 21*	370 $\pm$ 25*	306 $\pm$ 20*	281 $\pm$ 18*	163 $\pm$ 17*
CE/TC (%)	61.5	59.7	60.5	58.5	61.8	59.4

Fig. 2. Effects of leonurine on cholesterol efflux from human THP-1 macrophage-derived foam cells. After THP-1 cells were fully differentiated, cells were treated with different concentrations of leonurine (0, 5, 10, 20, 40, 80 μM) for 24 h. Then, apoA-I (10 μg/ml) or HDL (50 μg/ml) were added to the medium as lipid acceptors for another 6 h. Cellular cholesterol efflux was analyzed by liquid scintillation counting assays as described above. All results were obtained from three independent experiments, each performed in triplicate. Data were expressed as means ± SD. *P < 0.05 vs 0 μM group.

Fig. 3. Effects of leonurine on the expression of ABCA1 and ABCG1 in human THP-1 macrophages. After THP-1 cells were fully differentiated, cells were treated with different concentrations of leonurine (0, 5, 10, 20, 40, 80 μM) for 24 h. Then, western blot and RT-qPCR analyses were used to measure the protein (A, B) and mRNA (C, D) levels of ABCA1 and ABCG1. All results were obtained from three independent experiments, each performed in triplicate. Data were expressed as means ± SD. *P < 0.05 vs 0 μM group.

Effects of leonurine on the mRNA and protein levels of ABCA1 and ABCG1 in human THP-1 macrophages

It is well known that cholesterol efflux from peripheral cells is mainly mediated by some cholesterol transporters on the cell membrane, including ABCA1 and ABCG1. Next, we examined the effects of leonurine on ABCA1 and ABCG1 expressions to identify the mechanisms by which leonurine regulates lipid accumulation and cholesterol efflux from THP-1 macrophages. Cells (1×10^6 cells/well) were pre-treated with various concentrations of leonurine (0, 5, 10, 20, 40, 80 μM) for 24 h. Then, the mRNA and protein expression levels of ABCA1 and ABCG1 were quantified using RT-qPCR and western blot analyses, respectively. Results showed that leonurine significantly increased the expressions of ABCA1 and ABCG1

Fig. 4. LXRα is involved in the up-regulation of ABCA1 and ABCG1 induced by leonurine. A-C, THP-1 macrophage-derived foam cells were treated with different concentrations of leonurine (0, 5, 10, 20, 40, 80 μM) for 24 h. Then, RT-qPCR and western blot analyses were performed. *P < 0.05 vs 0 μM group. D-F, THP-1 macrophage-derived foam cells were transfected with LXRα and/or then treated with leonurine (80 μM) for 24 h. Then, RT-PCR and western blot analyses were performed. *P < 0.05 vs control group. #P < 0.05 vs leonurine only group. G-I, THP-1 macrophage-derived foam cells were divided into 4 groups and incubated with leonurine (80 μM) and/or T0901317 for 24 h. Then, RT-PCR and western blot analyses were performed. *P < 0.05 vs control group. #P < 0.05 vs leonurine only group. All results were obtained from three independent experiments, each performed in triplicate. Data were expressed as means ± SD.

at the mRNA and protein levels in a dose-dependent manner (Fig. 3), indicating that the positive effects of leonurine on cholesterol efflux from foam cells are attributed to the promotion of ABCA1 and ABCG1 expression.

The role of PPARγ/LXRα signaling in the effects of leonurine on ABCA1/G1 expression and cholesterol efflux in human THP-1 macrophages

Increasing evidence has shown that LXRs can modulate ABCA1 and ABCG1 expression, a process that influences cholesterol efflux and lipid content in macrophages [28, 29]. PPARγ is a common upstream factor of LXRs-induced signaling [30-32]. Next, we determined whether PPARγ/LXRα pathway is involved in the effects of leonurine on ABCA1/G1 expressions. Our data showed that leonurine potently up-regulated the mRNA and protein expression of LXRα in THP-1 macrophages-derived foam cells in a dose-dependent manner (Fig. 4A-C). Thereafter, cells were transfected with LXRα siRNA or incubated with LXR agonist T0901317, respectively. As shown in Fig. 4D-F, transfection of macrophages with LXRα siRNA decreased the mRNA and protein levels of LXRα, ABCA1 and ABCG1, and ruled out the enhancement of leonurine on LXRα, ABCA1/G1 expression. Meanwhile, cellular cholesterol efflux from macrophages incubated with the combination of LXRα siRNA and leonurine were potently decreased compared with those treated with leonurine alone (Fig. 6A-B). Then, we explored

Fig. 5. PPAR γ is involved in the up-regulation of ABCA1, ABCG1 and LXR α induced by leonurine. A-C, THP-1 macrophage-derived foam cells were treated with different concentrations of leonurine (0, 5, 10, 20, 40, 80 μ M) for 24 h. Then, RT-PCR and western blot analyses were performed. * $P < 0.05$ vs 0 μ M group. D-F, THP-1 macrophage-derived foam cells were transfected with PPAR γ siRNA and/or then treated with leonurine (80 μ M) for 24 h. Then, RT-PCR and western blot were performed. * $P < 0.05$ vs control group. # $P < 0.05$ vs leonurine only group. All results were obtained from three independent experiments, each performed in triplicate. Values were expressed as means $\pm$ SD.

Fig. 6. LXR α and PPAR γ are involved in the enhancement of cellular cholesterol efflux from human THP-1 macrophage-derived foam cells induced by leonurine. THP-1 macrophage-derived foam cells were transfected with LXR α siRNA or PPAR γ siRNA and/or incubated with leonurine (80 μ M) for 24 h. Then, cellular cholesterol efflux was analyzed by liquid scintillation counting assay as described in Methods. * $P < 0.05$ vs control group. # $P < 0.05$ vs leonurine only group. All results were obtained from three independent experiments, each performed in triplicate. Data were expressed as means $\pm$ SD.

the effects of LXR α agonist T0901317 on the amplification of ABCA1 and ABCG1 expressions. As indicated in Fig. 4G, up-regulation of ABCA1/G1 and LXR α mRNA expression by leonurine were significantly enhanced by the addition of T0901317. Similar results were observed using western blot analysis to detect the protein levels of ABCA1/G1 and LXR α (Fig. 4H-I).

Next, we further detected the effects of PPAR γ siRNA on the expressions of ABCA1/G1 and cholesterol efflux in leonurine-stimulated THP-1 macrophage-derived foam cells. As

Fig. 7. Effects of leonurine on the expressions of ABCA1/G1, LXRα, PPARγ, atherosclerotic lesions, and collagen contents of aortic roots in apoE^{-/-} mice. When apoE^{-/-} mice were 8 weeks old, they were intraperitoneally with PBS or leonurine (10 mg/kg/d) every day for 8 weeks. A and B, representative microscopic images of Oil Red O staining of aortic sinus lesion. Original magnification: ×40. C, quantification of the lesion area of mice (n=15/group). D and E, representative microscopic images of Masson's trichrome staining of aortic sinus lesion. Original magnification: ×40. F, quantification of the collagen contents of mice (n=15/group). Data were expressed as means ± SEM. G and H, ABCA1, ABCG1, LXRα and PPARγ levels were measured in the homogenate of aortic arch by western blot. *P < 0.05 vs control group. Values are mean ± SD (n = 3/group).

shown in Fig. 5 A-C, leonurine can also promote the mRNA and protein expressions of PPARγ in a concentration-dependent manner. Besides, transfection of foam cells with PPARγ siRNA significantly decreased LXRα, ABCA1 and ABCG1 mRNA and protein levels and ruled out the up-regulation of leonurine on LXRα, ABCA1 and ABCG1 expression (Fig. 5D-F). In line with these results, the enhancement of cellular cholesterol efflux by leonurine was almost fully compensated by transfection with PPARγ siRNA (Fig. 6C-D). These results indicated that PPARγ/LXRα signaling is involved in the stimulatory effects of leonurine on ABCA1/G1 expression and cholesterol efflux from human THP-1 macrophages.

Effects of leonurine on aortic atherosclerosis

Next, we evaluated the leonurine-induced atheroprotection and the potential mechanisms underlying its atheroprotective effects in apoE^{-/-} mice. As indicated in Fig. 7A-F, consistent with the observations in *ex vivo* experiments, the atherosclerotic lesion sizes were potently diminished in en face preparations of aortic roots in mice treated with leonurine, compared with the control group. Besides, the results revealed that both TG and TC levels in serum, were decreased in leonurine-treated animals. A detailed assessment of the plasma lipoproteins showed a decrease in LDL-C and an increase in HDL-C levels in mice injected with leonurine (Table 2). Finally, we analyzed the protein levels of PPARγ, LXRα, ABCA1

and ABCG1 in the tissue homogenate of aortic roots using western blot analysis. The results showed that the expressions of PPAR γ , LXR α , ABCA1 and ABCG1 were significantly increased in the aortic roots of leonurine-challenged mice compared with control mice (Fig. 7G-H). These outcomes suggest that leonurine impedes aortic lipid accumulation and regulates plasma lipoprotein cholesterol levels via promoting PPAR γ /LXR α -induced ABCA1 and ABCG1 expression.

Discussion

In the present study, we explored the atheroprotective effects of leonurine and the underlying mechanisms by which leonurine affects lipid accumulation and cholesterol efflux from THP-1 macrophages-derived foam cells and further atherogenesis in apoE $^{-/-}$ mice. The results showed that leonurine promotes macrophage ABCA1 and ABCG1 expression in a PPAR γ /LXR α signaling-dependent manner and stimulates ABCA1/G1-mediated cholesterol efflux from macrophage foam cells, leading to a diminution in cellular lipid and cholesterol accumulation. Moreover, leonurine remarkably decreased lipid deposition and atherosclerotic lesion sizes in aortic roots of apoE $^{-/-}$ mice. Fig. 8 is a graphic description of the mechanisms.

It is well received that the formation of atherosclerotic lesions, which is featured by mass deposition of macrophages-derived CE-rich foam cells within the arterial wall, is a major risk factor for atherogenesis [33, 34]. The abnormal homeostasis of cellular cholesterol is responsible for the development of AS. ABCA1 and ABCG1 are key cholesterol transporters that mediate the efflux of cellular phospholipids and FC to extracellular acceptors, resulting in HDL formation [35]. Here, our data extended a novel mechanism of the beneficial effects of leonurine on the progression of CVD. We identified that leonurine enhanced the expression of ABCA1 and ABCG1 and increased cholesterol efflux from THP-1 macrophage-derived foam cells. Besides, cellular cholesterol and CE levels were down-regulated in leonurine-treated macrophages. These findings suggest that leonurine-induced promotion in cellular cholesterol efflux is mainly ascribed to amplified expression of ABCA1 and ABCG1.

Leonurine (SCM-198) is the bioactive phenolic alkaloid ingredient of HL, which is generally called as "Yi-Mu-Cao". A large body of evidence has indicated that leonurine is effective in preventing many disorders through many ways, including stroke [36], chronic myocardial infarction [16, 37] and acute kidney injury [38]. Yuan et al. [39] found that leonurine hydrochloride (LH), an artificial chemical compound sharing the similar structure of leonurine, can protect against osteoclast-related diseases via inhibiting receptor activator NF- κ B ligand (RANKL)-TNF receptor-associated factor 6 (TRAF6) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways. Hong et al. [40] reported that leonurine can decrease the levels of TNF- α , nitric oxide (NO), IL-6 and interleukin-1 β IL-1 β) by inhibiting the activation of c-Jun N-terminal kinase (JNK) and NF- κ B, thus preventing microglial overactivation in rats. They also established that SCM-198 can enhance neuronal survival by stimulating brain-derived neurotrophic factor (BDNF)/tropomyosin-related kinase(TrkB)/cAMP-response element-binding protein (CREB) signaling in amyloid- β protein precursor and presenilin-1(A β PP/PS1) double-transgenic mice and therefore it might be used for Alzheimer's disease (AD) treatment [41]. Besides, Cheng et al. [42] identified leonurine as a candidate renoprotective remedy for the treatment of renal fibrosis by blocking the tumor growth factor (TGF- β)/Smad3 and NF- κ B pathway. Hitherto, many pharmaceutical studies

Table 2. Body weight and plasma lipid profile in apoE $^{-/-}$ mice. Plasma from various experimental groups was assessed enzymatically. Values were presented as means $\pm$ SD from the indicated numbers of apoE $^{-/-}$ male mice in each group. *P < 0.05 vs control group. TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; * compared with control group, p < 0.05

	Control (n=15)	Leonurine (n=15)
Body weight (g)	30.58 $\pm$ 2.15	29.12 $\pm$ 3.55
TG (mmol/L)	1.88 $\pm$ 0.45	1.25 $\pm$ 0.23*
TC (mmol/L)	17.59 $\pm$ 2.12	13.78 $\pm$ 1.85*
HDL-C (mmol/L)	0.95 $\pm$ 0.25	1.42 $\pm$ 0.43*
LDL-C (mmol/L)	10.54 $\pm$ 1.83	8.26 $\pm$ 1.35*

Fig. 8. Illustration of the mechanisms by which leonurine regulates cholesterol efflux through promoting ABCA1 and ABCG1 expression. Leonurine can stimulate PPAR γ -LXR α signaling, leading to the enhancement of ABCA1 and ABCG1 expression on cell membranes and subsequent increase in ABCA1-mediated cholesterol efflux to apoA-I and ABCG1-mediated cholesterol efflux to HDL.

have explored the cardioprotective effects of leonurine *in vivo* and *ex vivo*, most of which concentrate on its anti-inflammatory, anti-oxidative and anti-apoptotic functions [12, 43-46]. Our previous research has demonstrated that betulinic acid (BA) can promote ABCA1 expression and cholesterol efflux via inhibiting NF- κ B signaling and microRNA (miR)-33s [20]. These observations make us wonder whether leonurine can prevent atherogenesis through maintaining cellular cholesterol homeostasis. Our present study showed that treatment of lipid-loaded macrophages with leonurine significantly decreased lipid content and alleviated excessive cholesterol accumulation by amplifying ABCA1 and ABCG1 expression in concentration- dependent manner, supporting the beneficial role of leonurine in atherogenesis.

LXR α belongs to the LXR subfamily, which is closely associated with cholesterol and lipid metabolism, lipogenesis and carbohydrate metabolism [47]. Costet et al. [48] observed that the promoter activity of ABCA1 was increased in a sterol-responsive fashion after lipid-laden macrophages were cotransfected with LXR α /RXR. Further data identified approximately four nucleotides of the ABCA1 promoter (from -70 to -55 base pairs) as a binding site for LXR α /RXR. When this element was mutated, the augmented ABCA1 promoter activity induced by LXR α /RXR was abolished, suggesting that ABCA1 is one of the LXR α target genes. Furthermore, LXR α agonists can up-regulate the expression of ABCA1 and inhibit the differentiation of macrophages into foam cells by promoting cellular cholesterol efflux [48-52]. Similarly, LXR α activation can increase the transcription levels of ABCG1 in macrophages [51, 53], adipose tissue and liver [54, 55]. Many intervention studies in murine models have provided proofs that LXR α activators have the potential uses in AS. Treatment of either apoE $^{-/-}$ or LDLR $^{-/-}$ mice with LXR α agonist can reduce the lesion development to approximately 50% [56]. Further experiments showed that chronic administration of LXR α ligands can change the lipoprotein profile of these transgenic mice, indicating that the beneficial effects of LXR α ligands on cells of the artery wall are also involved in atheroprotection. In consistency with these observations, LXR α agonist was able to magnify the expression of ABCA1 and ABCG1 in the atherosclerotic aortas of apoE $^{-/-}$ mice [56]. In this study, we found that the leonurine can remarkably enhance LXR α levels in THP-1 macrophages-derived foam cells, and transfection of cells with LXR α siRNA abolished the positive effects of leonurine on ABCA1 and ABCG1 expression as wells as the cholesterol efflux. Similar results were also observed in leonurine-treated apoE $^{-/-}$ mice, suggesting that LXR α is involved in the leonurine-induced atheroprotection.

In liver, macrophages, adipose tissue and muscle, LXR α expression was modulated by nuclear receptors, particularly PPARs. PPAR γ is the key sensor of cellular fatty acids and sterol and plays critical roles in lipid homeostasis and inflammation in atherogenesis [57]. It is highly expressed in macrophage-derived lipid-rich foam cells of atheromatous lesions [58]. Tontonoz et al. [59] found that PPAR γ contributes to the formation of foam cells by stimulating CD36 expression and ox-LDL uptake. Additionally, two components of oxLDL, 9- and 13-HODE have been identified as PPAR γ ligands [60, 61]. In the aorta of LDL $^{-/-}$ male mice injected with PPAR γ specific agonists, however, plasma HDL levels were enhanced and the formation of atheromatous lesions was potently impeded despite the augmentation in CD36 expression [62]. Further analyses showed that PPAR γ can induce the expression of

ABCA1 and ABCG1 and promote cholesterol efflux from macrophages by targeting LXR α . Thus, PPAR γ seems to modulate both cholesterol influx and efflux for macrophages. Many studies have identified that PPAR γ agonists can induce LXR α expression in human and murine macrophages [63]. Accordingly, PPAR γ /LXR α signaling pathway is critical for the maintenance of lipid and glucose homeostasis, especially through the regulation of ABCA1/G1 expression. Chawla et al. [32] reported that the absence of macrophage PPAR γ -LXR α -ABCA1 pathway disrupts cholesterol efflux and aggravates atherogenesis *in vivo*. Ikhlef et al. [64] determined that oxLDL–paraoxonase 1 (PON1) potentially amplified cholesterol efflux through the PPAR γ -LXR α -ABCA1/G1 pathway in J774 macrophages compared to oxLDL alone. Besides, salvianolic acid B (Sal B) can also promote the efflux of excess cellular cholesterol from THP-1 cell lines by activating the PPAR γ -LXR α -ABCA1 pathway [65]. Consistent with these experiments, He et al. [66] observed that baicalin, a pharmaceutical extract from another Chinese herbal medicine, *Scutellaria baicalensis*, was able to alleviate lipid accumulation in macrophage-derived foam cells via stimulating the PPAR γ -LXR α -ABCA1/G1 pathway. Our study showed that leonurine can stimulate PPAR γ expression in THP-1 macrophages-derived foam cells and in aorta roots of apoE $^{-/-}$ mice. Moreover, transfection with PPAR γ siRNA into cells significantly ruled out the stimulatory effects of leonurine on the expression of LXR α , ABCA1 and ABCG1 as well as 3 H-cholesterol efflux. These results suggest that leonurine can exert atheroprotective effects through the PPAR γ -LXR α -ABCA1/G1 pathway.

In conclusion, our experiments, for the first time, provide evidence that leonurine can impede atherogenesis by relieving lipid accumulation and facilitating cholesterol efflux in lipid-laden macrophages, a process that is mediated by PPAR γ -LXR α -ABCA1/G1 signaling pathway. These findings may extend a molecular basis for the identification of leonurine as a novel pharmacological agent for the prevention and treatment of AS.

Disclosure Statement

There is no conflict of interests in this paper.

Acknowledgements

This work is fiscally supported by the National Natural Sciences Foundation of China (81660082), the Guangxi Natural Sciences Foundation (AA139209), Autonomous region College Students' innovation and entrepreneurship training program (201610601047), Hunan Provincial Innovation Foundation For Postgraduate (CX2017B503), Hunan Provincial Natural Science Foundation of China (14JJ2091); and Zhengxiang scholar program (prof. Xiangyang Tang) of the University of South China.

References

- 1 Weber C, Noels H: Atherosclerosis: Current pathogenesis and therapeutic options. *Nat Med* 2011;17:1410-1422.
- 2 Liang C, Wang X, Hu J, Lian X, Zhu T, Zhang H, Gu N, Li J: Ptpromotes oxidized low-density lipoprotein induced oxidative stress and cell apoptosis through toll-like receptor 4/nuclear factor kappaB pathway. *Cell Physiol Biochem* 2017;42:495-505.
- 3 Chistiakov DA, Orekhov AN, Bobryshev YV: Lox-1-mediated effects on vascular cells in atherosclerosis. *Cell Physiol Biochem* 2016;38:1851-1859.
- 4 Xu X, Zhang A, Li N, Li PL, Zhang F: Concentration-dependent diversification effects of free cholesterol loading on macrophage viability and polarization. *Cell Physiol Biochem* 2015;37:419-431.
- 5 Luo C, Lian X, Hong L, Zou J, Li Z, Zhu Y, Huang T, Zhang Y, Hu Y, Yuan H, Wen T, Zhuang W, Cai B, Zhang X, Hisatome I, Yamamoto T, Huang J, Cheng J: High uric acid activates the ros-ampk pathway, impairs cd68 expression and inhibits oxldl-induced foam-cell formation in a human monocytic cell line, thp-1. *Cell*

Physiol Biochem 2016;40:538-548.

- 6 Zhang BC, Zhang CW, Wang C, Pan DF, Xu TD, Li DY: Luteolin attenuates foam cell formation and apoptosis in ox-Ldl-stimulated macrophages by enhancing autophagy. *Cell Physiol Biochem* 2016;39:2065-2076.
- 7 Zhao GJ, Yin K, Fu YC, Tang CK: The interaction of apoA-I and abca1 triggers signal transduction pathways to mediate efflux of cellular lipids. *Mol Med* 2012;18:149-158.
- 8 Zhu X, Lee JY, Timmins JM, Brown JM, Boudyguina E, Mulya A, Gebre AK, Willingham MC, Hiltbold EM, Mishra N, Maeda N, Parks JS: Increased cellular free cholesterol in macrophage-specific abca1 knock-out mice enhances pro-inflammatory response of macrophages. *J Biol Chem* 2008;283:22930-22941.
- 9 Out R, Hoekstra M, Hildebrand RB, Kruit JK, Meurs I, Li Z, Kuipers F, Van Berkel TJ, Van Eck M: Macrophage abcg1 deletion disrupts lipid homeostasis in alveolar macrophages and moderately influences atherosclerotic lesion development in ldl receptor-deficient mice. *Arterioscler Thromb Vasc Biol* 2006;26:2295-2300.
- 10 Franccone OL, Royer L, Boucher G, Haghpasand M, Freeman A, Brees D, Aiello RJ: Increased cholesterol deposition, expression of scavenger receptors, and response to chemotactic factors in abca1-deficient macrophages. *Arterioscler Thromb Vasc Biol* 2005;25:1198-1205.
- 11 Yin K, Liao DF, Tang CK: Atp-binding membrane cassette transporter a1 (abca1): A possible link between inflammation and reverse cholesterol transport. *Mol Med* 2010;16:438-449.
- 12 Liu XH, Xin H, Zhu YZ: More than a "mother-benefiting" herb: Cardioprotective effect of herba leonuri. *Sheng Li Xue Bao* 2007;59:578-584.
- 13 Zheng L, Li Y, Li X, Kou J, Zhong Z, Jiang Y, Liu Z, Tian Y, Yang L: Combination of hydroxyl acetylated curcumin and ultrasound induces macrophage autophagy with anti-apoptotic and anti-lipid aggregation effects. *Cell Physiol Biochem* 2016;39:1746-1760.
- 14 Pang S, Tsuchiya S, Horie S, Uchida M, Murayama T, Watanabe K: Enhancement of phenylephrine-induced contraction in the isolated rat aorta with endothelium by h2o-extract from an oriental medicinal plant leonuri herba. *Jpn J Pharmacol* 2001;86:215-222.
- 15 Liu XH, Pan LL, Gong QH, Zhu YZ: Antiapoptotic effect of novel compound from herba leonuri - leonurine (scm-198): A mechanism through inhibition of mitochondria dysfunction in h9c2 cells. *Curr Pharm Biotechnol* 2010;11:895-905.
- 16 Liu X, Pan L, Gong Q, Zhu Y: Leonurine (scm-198) improves cardiac recovery in rat during chronic infarction. *Eur J Pharmacol* 2010;649:236-241.
- 17 Zhang Y, Guo W, Wen Y, Xiong Q, Liu H, Wu J, Zou Y, Zhu Y: Scm-198 attenuates early atherosclerotic lesions in hypercholesterolemic rabbits via modulation of the inflammatory and oxidative stress pathways. *Atherosclerosis* 2012;224:43-50.
- 18 Liu X, Pan L, Wang X, Gong Q, Zhu YZ: Leonurine protects against tumor necrosis factor-alpha-mediated inflammation in human umbilical vein endothelial cells. *Atherosclerosis* 2012;222:34-42.
- 19 Hao XR, Cao DL, Hu YW, Li XX, Liu XH, Xiao J, Liao DF, Xiang J, Tang CK: Ifn-gamma down-regulates abca1 expression by inhibiting Ixralpha in a jak/stat signaling pathway-dependent manner. *Atherosclerosis* 2009;203:417-428.
- 20 Zhao GJ, Tang SL, Lv YC, Ouyang XP, He PP, Yao F, Chen WJ, Lu Q, Tang YY, Zhang M, Fu Y, Zhang DW, Yin K, Tang CK: Antagonism of betulinic acid on lps-mediated inhibition of abca1 and cholesterol efflux through inhibiting nuclear factor-kappab signaling pathway and mir-33 expression. *PLoS One* 2013;8:e74782.
- 21 Chen WJ, Yin K, Zhao GJ, Fu YC, Tang CK: The magic and mystery of microrna-27 in atherosclerosis. *Atherosclerosis* 2012;222:314-323.
- 22 Ren K, Mo ZC, Liu X, Tang ZL, Jiang Y, Peng XS, Zhang QH, Shi JF, Yi GH: Tgf-beta down-regulates apolipoprotein m expression through the tak-1-jnk-c-jun pathway in hepg2 cells. *Lipids* 2017;52:109-117.
- 23 Ren K, Lu YJ, Mo ZC, Liu X, Tang ZL, Jiang Y, Peng XS, Li L, Zhang QH, Yi GH: ApoA-I/sr-bi modulates s1p/s1pr2-mediated inflammation through the pi3k/akt signaling pathway in huvecs. *J Physiol Biochem* 2017
- 24 Liu XY, Lu Q, Ouyang XP, Tang SL, Zhao GJ, Lv YC, He PP, Kuang HJ, Tang YY, Fu Y, Zhang DW, Tang CK: Apelin-13 increases expression of atp-binding cassette transporter a1 via activating protein kinase c alpha signaling in thp-1 macrophage-derived foam cells. *Atherosclerosis* 2013;226:398-407.
- 25 Lv YC, Tang YY, Peng J, Zhao GJ, Yang J, Yao F, Ouyang XP, He PP, Xie W, Tan YL, Zhang M, Liu D, Tang DP, Cayabyab FS, Zheng XL, Zhang DW, Tian GP, Tang CK: Microrna-19b promotes macrophage cholesterol accumulation and aortic atherosclerosis by targeting atp-binding cassette transporter a1. *Atherosclerosis* 2014;236:215-226.

- 26 Hu YW, Ma X, Li XX, Liu XH, Xiao J, Mo ZC, Xiang J, Liao DF, Tang CK: Eicosapentaenoic acid reduces abca1 serine phosphorylation and impairs abca1-dependent cholesterol efflux through cyclic amp/protein kinase a signaling pathway in thp-1 macrophage-derived foam cells. *Atherosclerosis* 2009;204:e35-43.
- 27 Zhao GJ, Mo ZC, Tang SL, Ouyang XP, He PP, Lv YC, Yao F, Tan YL, Xie W, Shi JF, Wang Y, Zhang M, Liu D, Tang DP, Zheng XL, Tian GP, Tang CK: Chlamydia pneumoniae negatively regulates abca1 expression via tlr2-nuclear factor-kappa b and mir-33 pathways in thp-1 macrophage-derived foam cells. *Atherosclerosis* 2014;235:519-525.
- 28 Hu YW, Wang Q, Ma X, Li XX, Liu XH, Xiao J, Liao DF, Xiang J, Tang CK: Tgf-beta1 up-regulates expression of abca1, abcg1 and sr-bi through liver x receptor alpha signaling pathway in thp-1 macrophage-derived foam cells. *J Atheroscler Thromb* 2010;17:493-502.
- 29 Nelson JK, Koenis DS, Scheij S, Cook EC, Moeton M, Santos A, Lobaccaro JA, Baron S, Zelcer N: Eepd1 is a novel lxr target gene in macrophages which regulates abca1 abundance and cholesterol efflux. *Arterioscler Thromb Vasc Biol* 2017;37:423-432.
- 30 Bastias-Candia S, Garrido AN, Zolezzi JM, Inestrosa NC: Recent advances in neuroinflammation therapeutics: Ppars/lxr as neuroinflammatory modulators. *Curr Pharm Des* 2016;22:1312-1323.
- 31 Xu X, Li Q, Pang L, Huang G, Huang J, Shi M, Sun X, Wang Y: Arctigenin promotes cholesterol efflux from thp-1 macrophages through ppar-gamma/lxr-alpha signaling pathway. *Biochem Biophys Res Commun* 2013;441:321-326.
- 32 Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM, Tontonoz P: A ppar gamma-lxr-abca1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol Cell* 2001;7:161-171.
- 33 Yang X, Yu Y, Wang D, Qin S: Overexpressed pltp in macrophage may promote cholesterol accumulation by prolonged endoplasmic reticulum stress. *Med Hypotheses* 2017;98:45-48.
- 34 Yao Y, Yang D, Han Y, Wang W, Wang N, Yang J, Zeng C: Dopamine d1-like receptors suppress the proliferation of macrophages induced by ox-ldl. *Cell Physiol Biochem* 2016;38:415-426.
- 35 Fitzgerald ML, Mujawar Z, Tamehiro N: Abc transporters, atherosclerosis and inflammation. *Atherosclerosis* 2010;211:361-370.
- 36 Loh KP, Qi J, Tan BK, Liu XH, Wei BG, Zhu YZ: Leonurine protects middle cerebral artery occluded rats through antioxidant effect and regulation of mitochondrial function. *Stroke* 2010;41:2661-2668.
- 37 Liu XH, Pan LL, Chen PF, Zhu YZ: Leonurine improves ischemia-induced myocardial injury through antioxidative activity. *Phytomedicine* 2010;17:753-759.
- 38 Xu D, Chen M, Ren X, Ren X, Wu Y: Leonurine ameliorates lps-induced acute kidney injury via suppressing ros-mediated nf-kappab signaling pathway. *Fitoterapia* 2014;97:148-155.
- 39 Yuan FL, Xu RS, Jiang DL, He XL, Su Q, Jin C, Li X: Leonurine hydrochloride inhibits osteoclastogenesis and prevents osteoporosis associated with estrogen deficiency by inhibiting the nf-kappab and pi3k/akt signaling pathways. *Bone* 2015;75:128-137.
- 40 Hong ZY, Shi XR, Zhu K, Wu TT, Zhu YZ: Scm-198 inhibits microglial overactivation and attenuates abeta(1-40)-induced cognitive impairments in rats via jnk and nf-small ka, cyrillicb pathways. *J Neuroinflammation* 2014;11:147.
- 41 Hong ZY, Yu SS, Wang ZJ, Zhu YZ: Scm-198 ameliorates cognitive deficits, promotes neuronal survival and enhances creb/bdnf/trkb signaling without affecting abeta burden in abetapp/ps1 mice. *Int J Mol Sci* 2015;16:18544-18563.
- 42 Cheng H, Bo Y, Shen W, Tan J, Jia Z, Xu C, Li F: Leonurine ameliorates kidney fibrosis via suppressing tgf-beta and nf-kappab signaling pathway in uuo mice. *Int Immunopharmacol* 2015;25:406-415.
- 43 Liu C, Guo W, Shi X, Kaium MA, Gu X, Zhu YZ: Leonurine-cysteine analog conjugates as a new class of multifunctional anti-myocardial ischemia agent. *Eur J Med Chem* 2011;46:3996-4009.
- 44 Liu XH, Chen PF, Pan LL, Silva RD, Zhu YZ: 4-guanidino-n-butyl syringate (leonurine, scm 198) protects h9c2 rat ventricular cells from hypoxia-induced apoptosis. *J Cardiovasc Pharmacol* 2009;54:437-444.
- 45 Liu H, Zhang X, Du Y, Ji H, Li S, Li L, Xing Y, Zhang X, Dong L, Wang C, Zhao K, Ji Y, Cao X: Leonurine protects brain injury by increased activities of ucp4, sod, cat and bcl-2, decreased levels of mda and bax, and ameliorated ultrastructure of mitochondria in experimental stroke. *Brain Res* 2012;1474:73-81.
- 46 Gao H, Yang X, Gu X, Zhu YZ: Synthesis and biological evaluation of the codrug of leonurine and aspirin as cardioprotective agents. *Bioorg Med Chem Lett* 2016;26:4650-4654.

- 47 Geyeregger R, Zeyda M, Stulnig TM: Liver x receptors in cardiovascular and metabolic disease. *Cell Mol Life Sci* 2006;63:524-539.
- 48 Costet P, Luo Y, Wang N, Tall AR: Sterol-dependent transactivation of the abc1 promoter by the liver x receptor/retinoid x receptor. *J Biol Chem* 2000;275:28240-28245.
- 49 Laffitte BA, Repa JJ, Joseph SB, Wilpitz DC, Kast HR, Mangelsdorf DJ, Tontonoz P: Lxrs control lipid-inducible expression of the apolipoprotein e gene in macrophages and adipocytes. *Proc Natl Acad Sci U S A* 2001;98:507-512.
- 50 Schwartz K, Lawn RM, Wade DP: Abc1 gene expression and apoa-i-mediated cholesterol efflux are regulated by lxr. *Biochem Biophys Res Commun* 2000;274:794-802.
- 51 Venkateswaran A, Repa JJ, Lobaccaro JM, Bronson A, Mangelsdorf DJ, Edwards PA: Human white/murine abc8 mRNA levels are highly induced in lipid-loaded macrophages. A transcriptional role for specific oxysterols. *J Biol Chem* 2000;275:14700-14707.
- 52 Venkateswaran A, Laffitte BA, Joseph SB, Mak PA, Wilpitz DC, Edwards PA, Tontonoz P: Control of cellular cholesterol efflux by the nuclear oxysterol receptor lxr alpha. *Proc Natl Acad Sci U S A* 2000;97:12097-12102.
- 53 Tangirala RK, Bischoff ED, Joseph SB, Wagner BL, Walczak R, Laffitte BA, Daige CL, Thomas D, Heyman RA, Mangelsdorf DJ, Wang X, Lusis AJ, Tontonoz P, Schulman IG: Identification of macrophage liver x receptors as inhibitors of atherosclerosis. *Proc Natl Acad Sci U S A* 2002;99:11896-11901.
- 54 Nakamura K, Kennedy MA, Baldan A, Bojanic DD, Lyons K, Edwards PA: Expression and regulation of multiple murine atp-binding cassette transporter g1 mRNAs/isoforms that stimulate cellular cholesterol efflux to high density lipoprotein. *J Biol Chem* 2004;279:45980-45989.
- 55 Ulven SM, Dalen KT, Gustafsson JA, Nebb HI: Tissue-specific autoregulation of the lxralpha gene facilitates induction of apoe in mouse adipose tissue. *J Lipid Res* 2004;45:2052-2062.
- 56 Joseph SB, McMilligan E, Pei L, Watson MA, Collins AR, Laffitte BA, Chen M, Noh G, Goodman J, Hagger GN, Tran J, Tipping TK, Wang X, Lusis AJ, Hsueh WA, Law RE, Collins JL, Willson TM, Tontonoz P: Synthetic lxr ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci U S A* 2002;99:7604-7609.
- 57 Majdalawieh A, Ro HS: Ppargamma1 and lxralpha face a new regulator of macrophage cholesterol homeostasis and inflammatory responsiveness, aebp1. *Nucl Recept Signal* 2010;8:e004.
- 58 Willson TM, Lambert MH, Kliewer SA: Peroxisome proliferator-activated receptor gamma and metabolic disease. *Annu Rev Biochem* 2001;70:341-367.
- 59 Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM: Ppargamma promotes monocyte/macrophage differentiation and uptake of oxidized ldl. *Cell* 1998;93:241-252.
- 60 Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM: Oxidized ldl regulates macrophage gene expression through ligand activation of ppargamma. *Cell* 1998;93:229-240.
- 61 Huang JT, Welch JS, Ricote M, Binder CJ, Willson TM, Kelly C, Witztum JL, Funk CD, Conrad D, Glass CK: Interleukin-4-dependent production of ppar-gamma ligands in macrophages by 12/15-lipoxygenase. *Nature* 1999;400:378-382.
- 62 Li AC, Brown KK, Silvestre MJ, Willson TM, Palinski W, Glass CK: Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in ldl receptor-deficient mice. *J Clin Invest* 2000;106:523-531.
- 63 Chinetti G, Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, Teissier E, Minnich A, Jaye M, Duverger N, Brewer HB, Fruchart JC, Clavey V, Staels B: Ppar-alpha and ppar-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the abca1 pathway. *Nat Med* 2001;7:53-58.
- 64 Ikhlef S, Berrougui H, Kamtchueng Simo O, Khalil A: Paraoxonase 1-treated oxldl promotes cholesterol efflux from macrophages by stimulating the ppargamma-lxralpha-abca1 pathway. *FEBS Lett* 2016;590:1614-1629.
- 65 Yue J, Li B, Jing Q, Guan Q: Salvianolic acid b accelerated abca1-dependent cholesterol efflux by targeting ppar-gamma and lxralpha. *Biochem Biophys Res Commun* 2015;462:233-238.
- 66 He XW, Yu D, Li WL, Zheng Z, Lv CL, Li C, Liu P, Xu CQ, Hu XF, Jin XP: Anti-atherosclerotic potential of baicalin mediated by promoting cholesterol efflux from macrophages via the ppargamma-lxralpha-abca1/abcg1 pathway. *Biomed Pharmacother* 2016;83:257-264.