

Allicin induces the upregulation of ABCA1 expression via PPAR γ /LXR α signaling in THP-1 macrophage-derived foam cells

XIAO-LONG LIN^{1*}, HUI-JUN HU¹, YUAN-BO LIU^{2*}, XUE-MEI HU¹, XIAO-JUAN FAN¹, WEI-WEN ZOU¹, YONG-QUAN PAN¹, WEN-QUAN ZHOU¹, MIN-WEN PENG¹ and CAI-HONG GU¹

¹Department of Pathology, Huizhou Third People's Hospital, Guangzhou Medical University, Huizhou, Guangdong 516002; ²Medical Department of Neurology, The Sixth People's Hospital of Huizhou (The People's Hospital of Huiyang), Huizhou, Guangdong 516211, P.R. China

Received December 19, 2015; Accepted April 4, 2017

DOI: 10.3892/ijmm.2017.2949

Abstract. Allicin is considered anti-atherosclerotic due to its antioxidant and anti-inflammatory effects, which makes it an important drug for the prevention and treatment of atherosclerosis. However, the effects of allicin on foam cells are unclear. Thus, in this study, we examined the effects of allicin on lipid accumulation via peroxisome proliferator-activated receptor γ (PPAR γ)/liver X receptor α (LXR α) in THP-1 macrophage-derived foam cells. THP-1 cells were exposed to 100 nM phorbol myristate acetate (PMA) for 24 h, and then to oxidized low-density lipoprotein (ox-LDL; 50 mg/ml) to induce foam cell formation. The results of Oil Red O staining and high-performance liquid chromatography (HPLC) revealed showed that pre-treatment of the foam cells with allicin decreased total cholesterol, free cholesterol (FC) and cholesterol ester levels in cells, and also decreased lipid accumulation. Moreover, allicin upregulated ATP binding cassette transporter A1 (ABCA1) expression and promoted cholesterol efflux. However, these effects were significantly abolished by transfection with siRNA targeting ABCA1. Furthermore, PPAR γ /LXR α signaling was activated by allicin treatment. The allicin-induced upregulation of ABCA1 expression was also abolished by PPAR γ inhibitor (GW9662) and siRNA or LXR α siRNA co-treatment. Overall, our data demonstrate that the allicin-induced upregulation of ABCA1 promotes cholesterol efflux and reduces lipid accumulation via PPAR γ /LXR α signaling in THP-1 macrophage-derived foam cells.

Introduction

Atherosclerotic cardiovascular diseases are among the major causes of health issues in the past decades. Macrophages, particularly foam cells, play a pivotal role in the development and progression of early- and late-stage atherosclerotic lesions (1). Foam cell development is related to the imbalance between lipid uptake and efflux (2). Promoting cholesterol efflux from foam cells is important to decrease the size of atherosclerotic plaques and protect the cells from atherosclerosis. Multiple mechanisms are involved in cholesterol efflux, including free diffusion, membrane adenosine triphosphate (ATP)-binding cassette (ABC) transporters and lipoprotein receptors (3,4).

ABCA1 is a key transporter which mediates cellular cholesterol and phospholipid efflux to lipid-poor apolipoprotein A-I (apoA-I) in high-density lipoprotein (HDL) synthesis and reverses cholesterol transport, which is underscored by the marked accumulation of lipids in peripheral tissues observed in Tangier disease (5). Furthermore, loss-of-function mutations in the ABCA1 gene in humans positively correlate with aortic intima thickness (6). ABCA1 mutations block the capacity of ABCA1 to induce macrophage cholesterol efflux and have been shown to promote atherosclerosis (7). Thus, ABCA1 is thought to be a promising therapeutic target for the prevention of atherosclerosis.

The expression of the ABCA1 gene in macrophages is transcriptionally regulated by ligand-dependent nuclear receptors (8). Peroxisome proliferator-activated receptor γ (PPAR γ) (a member of the nuclear receptor superfamily) enhances cholesterol efflux by inducing the transcription of the liver X receptor α (LXR α) gene and ABCA1 (9). LXR α binds heterodimers with retinoid X receptor and follows a connection of specific DNA response elements in the ABCA1 promoter to stimulate the ABCA1 gene transcription. This stimulation increases ABCA1-dependent cholesterol efflux to apoA-I (10). Thus, PPAR γ /LXR α /ABCA1 signaling represents a powerful means of stimulating cholesterol efflux in macrophages and strongly affects the development of atherosclerotic plaques.

Allicin is an essential anti-atherosclerotic that has been studied for its cardioprotective properties with very promising results (11). A recent study reported that allicin exerts powerful

Correspondence to: Dr Xiao-Long Lin, Department of Pathology, Huizhou Third People's Hospital, Guangzhou Medical University, Huizhou, Guangdong 516002, P.R. China
E-mail: 493814078@qq.com

*Contributed equally

Key words: allicin, atherosclerosis, ATP binding cassette transporter A1, foam cells

effects, protecting HUVECs from apoptosis and suggested that the protection occurs via a mechanism involving the protection from H₂O₂-mediated oxidative stress (12). In addition, alliin reduces cholesterol levels and inhibits macrophage cytokine production induced by lipopolysaccharide (LPS), and alters the composition of fatty acids in mice or rats fed a high-fat acid diet (13,14). However, the role of alliin in lipid accumulation in foam cells remains unclear.

The present study explored the effects of alliin on lipid accumulation and cholesterol metabolism-related gene expression in order to examine the effects of cholesterol efflux on THP-1 macrophage-derived foam cells. Our findings demonstrate that alliin reduces lipid accumulation through the upregulation of ABCA1 expression via PPAR γ /LXR α signaling in THP-1 macrophage-derived foam cells. Overall, the results provide a new direction for the prevention of atherosclerosis.

Materials and methods

Materials and reagents. RPMI-1640 medium (SH30809) and fetal bovine serum (FBS; SH30088.03HI) were acquired from (GE Healthcare Life Sciences HyClone, Logan, UT, USA); TRIzol reagent (15596026), Lipofectamine[®] 2000 transfection reagent (11668030) and the cDNA synthesis kit (N8080234) were purchased from Invitrogen/Thermo Fisher Scientific, Inc. (Waltham, MA, USA); antibodies to alliin (sc-480646), phorbol myristate acetate (PMA; sc-3576) and GW9662 (PPAR γ antagonist; sc-202641), rabbit monoclonal antibody against ABCA1 (sc-53482), and β -actin (sc-7210), LXR α (sc-1000) and PPAR γ (sc-9000) antibodies were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); cell lysis buffer (P0013) was purchased from Beijing ComWin Biotech Co., Ltd. (Beijing, China); streptomycin and penicillin (ST488-1 and ST488-2) were purchased from Beyotime (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; C0009) was obtained from Beyotime.

Construction of foam cell model. THP-1 cells (purchased from the Cell Culture Center, Institute of Biochemistry and Cell Biology, Chinese Academy of Life Sciences, Shanghai, China; cat. no. CBD11410) were cultured in RPMI-1640 (10% FBS, 100 μ g/ml streptomycin and 100 U/ml penicillin) at 37°C in a 5% CO₂ humidified atmosphere, and then treated 100 nM PMA for 24 h. Subsequently, the medium was replaced with fresh medium and the cells were incubated with 50 mg/ml oxidized low-density lipoprotein (ox-LDL) for 48 h to establish the model of THP-1 macrophage-derived foam cells.

High-performance liquid chromatography (HPLC) assays. HPLC analysis was conducted as previously described (15). Sterol analyses were performed using a HPLC system (2790; controlled with Empower Pro software; Waters Corp., Milford, MA, USA). Sterols were detected using a photodiode array detector equipped with a 4⁻¹ liter cell (996; Waters Corp.). The analysis of cholesterol and cholesterol esters was performed following elution with acetonitrile (sc-477507)-isopropanol (sc-489314) (both from Santa Cruz Biotechnology, Inc.) at 30:70 (v/v) and detected by absorbance at 210 nm.

Cellular cholesterol efflux experiments. Cellular cholesterol efflux analysis was conducted as previously described (15). In brief, the THP-1 cells were cultured with 0.2 μ l Ci/ml of [³H]cholesterol and ox-LDL (50 μ g/ml) for 48 h, followed by treatment with alliin. The cells were then washed with phosphate-buffered saline (PBS) 3 times and incubated with RPMI-1640 medium containing 0.1% BSA and 20 μ g/ml human plasma apoA-1 (sc-111827; Santa Cruz Biotechnology, Inc.) overnight. Liquid scintillation counting was used to measure [³H]cholesterol in the medium and cells. Percentage efflux was calculated using the following equation: [total medium counts/(total cellular counts + total medium counts)] x100%.

Evaluation of lipid accumulation by Oil Red O staining. THP-1 macrophage-derived foam cells were seeded in 6-well plates (4x10⁵ cells/well). The cells were treated with alliin, alone or together with small interfering RNA (siRNA) or GW9662 for an additional 6 h. After 6 h, the cells were washed with PBS 3 times (15 sec each time), and incubated with 10% formalin 5 min. After rinsing with 60% isopropanol, the cells incubated with fresh filtered Oil Red O solution for 15 min and then washed with isopropanol (60%) (sc-489314), followed by counterstaining with hematoxylin (sc-396328) (both from Santa Cruz Biotechnology, Inc.) for 4 min and the cells were then observed and photographed using a microscope.

MTT assay. The THP-1 macrophage-derived foam cells (8x10³/ml) were seeded in 96-well microtiter plates (CW0543; ComWin Biotech Co., Ltd., Beijing, China). The cells were then incubated with various concentrations of alliin (2.5, 5, 10, 20 and 40 g/l) for 24 h or were incubated with 5 g/l alliin for different time periods of time (3, 6, 12, 24 and 48 h). Subsequently, 10 μ l MTT solution was added to each well, followed by incubation for 4 h at 37°C. The absorbance at the 490 nm wavelength was measured using a 2104 EnVision Multilabel Reader (cat. no. 2104-0010; PerkinElmer, Inc., Waltham, MA, USA). Cell viability was calculated as follows: cell viability (%) = [(alliin A value - untreated control A value)/[(control group A value - untreated control A value)] x100%.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using TRIzol reagent (cat. no. 15596026; Invitrogen/Thermo Fisher Scientific, Inc.). Subsequently, complementary DNA was synthesized using a reverse transcriptase kit (cat. no. N8080234; Invitrogen/Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The relative mRNA expression levels of were determined using a SYBR-Green real-time PCR kit (cat. no. 4367659; Agilent Technologies, Inc., Santa Clara, CA, USA) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). qPCR was performed using the ABI 7500 Fast Real-Time PCR system (cat. no. 4406985; Applied Biosystems/Thermo Fisher Scientific, Inc.) and the following gene-specific primers: GAPDH sense, 5'-TGCCATCAACGACCCCTTCA-3' and antisense, 5'-TGACCTTGCCCACAGCC TTG-3'; ABCA1 sense, 5'-TCCAGGCCAGTACGGAATTC-3' and antisense, 5'-ACTTTCCCTCGCCAAACCAGTAG-3'; LXR α sense, 5'-TCTGCGGTGGAGCTGTGGAA-3' and antisense, 5'-TGACGCTGGCGGAAGAAT-3'; PPAR γ sense,

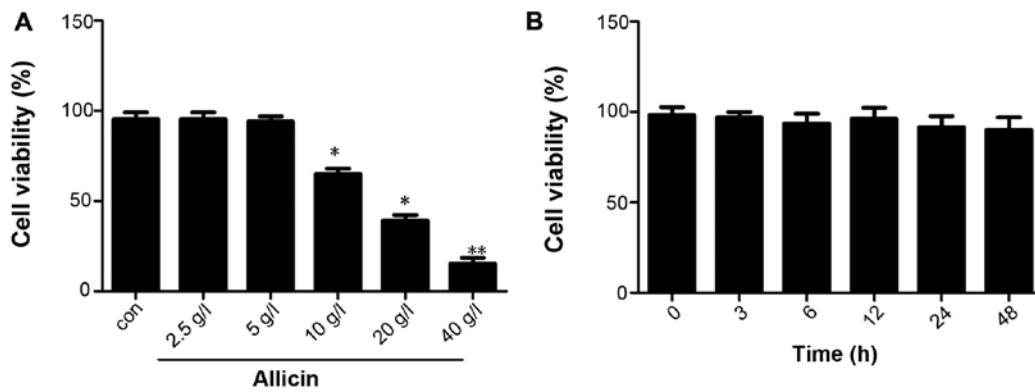


Figure 1. Effect of allicin at various concentrations on the viability of THP-1 macrophage-derived foam cells. (A) THP-1 macrophage-derived foam cells were stimulated with PMA and ox-LDL (50 mg/l), and then treated with 2.5, 5, 10, 20 and 40 g/l allicin for 24 h and were subjected to MTT assay to examine cell viability. (B) THP-1 macrophage-derived foam cells were stimulated with PMA and ox-LDL (50 mg/l), and then treated with 5 g/l allicin for 3, 6, 12, 24 and 48 h and were subjected to MTT assay to examine cell viability. The results are representative of 3 independent experiments. Data are expressed as the means \pm SD (n=3), *P<0.05 and **P<0.01 vs. control. ox-LDL, oxygenized low density lipoprotein; PMA, phorbol myristate acetate.

5'-CCTCCCTGATGAATAAAGATGG-3' and antisense, 5'-GCAAACCTCAAACCTTAGGCTCCA-3'. All primers were designed using the National Center for Biotechnology Information Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=Blast Home). PCR was performed under the following conditions: denaturation at 50°C for 2 min, followed by 38 cycles of 95°C for 15 sec and 60°C for 1 min. Gene expression was normalized to internal controls and fold changes were calculated using relative quantification ($2^{-\Delta\Delta Cq}$).

Western blot analysis. The cells were lysed in RIPA buffer (cat. no. P0013; Beyotime) and 1 mmol/l phenyl methyl sulfonyl fluoride (PMSF; cat. no. ST506-2; Beyotime) at 94:6. The protein concentration was determined using a BCA protein assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (cat. no. P0012A) (10%) and then transferred onto a polyvinylidene difluoride membranes (PVDF) (cat. no. FFP39) (both from Beyotime). The membranes were immunoblotted with anti- β -actin (1:1,000), anti-ABCA1 (1:500), anti-PPAR γ (1:250), anti-LXR α (1:250) antibodies at 4°C overnight. Subsequently, the corresponding secondary antibody (1:1,000) conjugated with peroxidase and enhanced chemiluminescence reagents (cat. no. P0018; Beyotime) were applied to visualize the targeted antigens. The protein contents were assessed using LabWork image analysis software (cat. no. P2403; Biomagin Systems Pvt., Ltd., Battaramulla, Sri Lanka).

Transfection with siRNA. siRNA targeting ABCA1 (Q000000019-1-B) were purchased from RiboBio Co., Ltd. (Guangzhou, China), PPAR γ (sc-29455) and LXR α (sc-38829) were all purchased from Santa Cruz Biotechnology, Inc. A control siRNA specific for the red fluorescent protein (CCACTACCTGAGCACCCAG) was used as a negative control (sc-37007; Santa Cruz Biotechnology, Inc.). The cells (2×10^6 cells/well) were transfected using Lipofectamine 2000 (Invitrogen) as previously described (16). The efficiency of transfection was examined by RT-qPCR and western blot analysis.

Statistical analysis. The experiments were performed in 3 or more different repetitions. The data are presented as the means \pm standard deviation (SD). The statistical significance of differences between groups was analyzed with the Student's t-test using SPSS 11.0 and GraphPad Prism 5.0 software. Values of P \leq 0.05 were considered to indicate statistically significant differences.

Results

Effect of allicin on the viability of THP-1 macrophage-derived foam cells. First, we examined the effect of allicin on THP-1 macrophage-derived foam cells. THP-1 macrophage-derived foam cells were stimulated with PMA and ox-LDL (50 mg/l), and the cells were then maintained in fresh serum-free medium for 4 h to synchronize their growth. The medium was replaced with fresh serum-free medium containing various concentrations of allicin (2.5, 5, 10, 20 and 40 g/l) and the cells were then incubated for 24 h. The results of MTT assay indicated that the viability of THP-1 macrophage-derived foam cells decreased with the increasing concentrations of allicin, with the most prominent effect observed at the concentration of 40 g/l; cell viability was not altered at a low concentration of allicin (5 g/l) (Fig. 1A). The THP-1 macrophage-derived foam cells were incubated with 5 g/l allicin for 0, 6, 12, 24 and 48 h to investigate whether allicin reduces cell viability in a time-dependent manner. The results of MTT assay indicated that the viability of the THP-1 macrophage-derived foam cells was not altered when incubated with 5 g/l allicin for different periods of time (Fig. 1B).

Allicin reduces lipid accumulation in THP-1 macrophage-derived foam cells. The THP-1 macrophage-derived foam cells were incubated with 5 g/l of allicin for 24 h to investigate whether allicin reduces lipid accumulation. The cells were stained with Oil Red O. Compared with the model group, 5 g/l allicin significantly decreased intracellular lipid droplet accumulation (Fig. 2). Moreover, we detected the total cholesterol (TC), free cholesterol (FC), and cholesterol ester (CE) levels in THP-1 macrophage-derived foam cells following incubation with 5 g/l allicin for 24 h. The results revealed that

Table I. Effects of allixin on free cholesterol and cholesterol esters in THP-1 macrophage-derived foam cells.

Group	TC	FC	CE	CE/TC (%)
Control	152.32±11.54	98.73±7.29	55.57±5.61	36.48
Model group	513.75±31.46 ^a	186.48±21.16 ^a	327.23±23.12 ^a	63.69 ^a
Allixin (5 g/l)	176.98±20.56 ^b	91.18±25.62 ^b	85.92±24.39 ^b	48.54 ^b

THP-1 macrophage-derived foam cells were divided into 3 groups and cultured at 37°C in medium containing 5 g/l allixin for 12 h. Cellular cholesterol and CE were extracted, as described in the Materials and methods. HPLC was performed to determine the levels of cellular TC, FC and CE. Results are expressed as the means ± SD of 3 independent experiments performed in triplicate. ^aP<0.05 is used in the comparison with the control group; ^bP<0.05 is used in the comparison with the model group. TC, total cholesterol; FC, free cholesterol; CE, cholesterol ester; HPLC, high-performance liquid chromatography. Data are the means ± SD, n=3. Cholesterol levels are in mg/g.

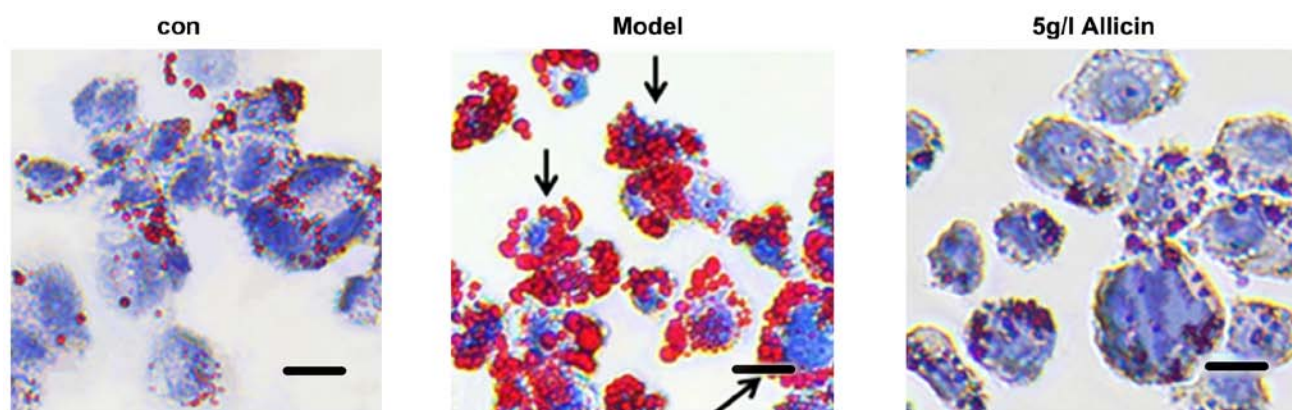


Figure 2. Effect of allixin on lipid droplet accumulation in THP-1 macrophage-derived foam cells. The cells were stimulated with PMA and ox-LDL (50 mg/l) and then incubated with 5 g/l of allixin for 24 h. Subsequently, the cells were stained with Oil Red O. Scale bar, 10 μm. Magnification, x200. Black arrows indicate foam cells. ox-LDL, oxygenized low density lipoprotein; PMA, phorbol myristate acetate.

allixin significantly decreased the levels of TC, FC and CE in the THP-1 macrophage-derived foam cells (Table I). These findings demonstrated that allixin reduced lipid accumulation in THP-1 macrophage-derived foam cells.

Allixin increases cholesterol efflux by upregulating ABCA1 expression in THP-1 macrophage-derived foam cells. Reverse cholesterol transport (RCT) is the key to inhibit the formation of foam cells. ABCA1, a membrane transporter, plays a critical role in cholesterol efflux, HDL metabolism and macrophage RCT (16). Therefore, in this study, we first examined the effects of allixin on cholesterol efflux. The results revealed that allixin increased cholesterol efflux in THP-1 macrophage-derived foam cells (Table II). The mRNA and protein expression patterns of ABCA1 were detected by RT-qPCR and western blot analysis. As shown in Fig. 3, allixin significantly upregulated ABCA1 expression at both the mRNA and protein level.

Subsequently, we further investigated whether allixin increases cholesterol efflux by upregulating the ABCA1 expression in THP-1 macrophage-derived foam cells. Transfection with ABCA1 siRNA eliminated the effects of allixin, thus increasing intracellular lipid droplet accumulation, leading to higher levels of TC, FC and CE in the transfected cells compared to the cells treated with allixin (Fig. 4 and Table III). Furthermore, we detected the

protein expression of PPARγ and LXRα. As shown in Fig. 3B, allixin increased PPARγ and LXRα protein expression, indicating that the upregulation of ABCA1 expression occurred via PPARγ/LXRα signaling.

PPARγ-LXRα signaling is involved in the allixin-induced upregulation of ABCA1 expression in THP-1 macrophage-derived foam cells. Previous studies have reported that PPARγ/LXRα signaling is the key to upregulating ABCA1 expression (7,10). Thus, we wished to further confirm whether allixin upregulates the expression of ABCA1 via PPARγ/LXRα signaling in THP-1 macrophage-derived foam cells. Firstly, the THP-1 macrophage-derived foam cells were treated with PPARγ siRNA or GW9662 (a PPARγ antagonist; 10 mmol/l) prior to exposure to 5 g/l allixin. As shown in Fig. 5, pre-treatment of the cells with PPARγ siRNA or GW9662 markedly abolished the effects of allixin, leading to a decrease in the expression of LXRα and ABCA1. These results indicate that PPARγ is involved in the allixin-induced upregulation of ABCA1 expression, and that LXRα may play a role in the regulation of ABCA1 expression by allixin. Moreover, transfection of the THP-1 macrophage-derived foam cells with LXRα siRNA significantly decreased the expression of ABCA1 (Fig. 6). These results thus indicate that allixin upregulates ABCA1 expression via PPARγ/LXRα signaling in THP-1 macrophage-derived foam cells.

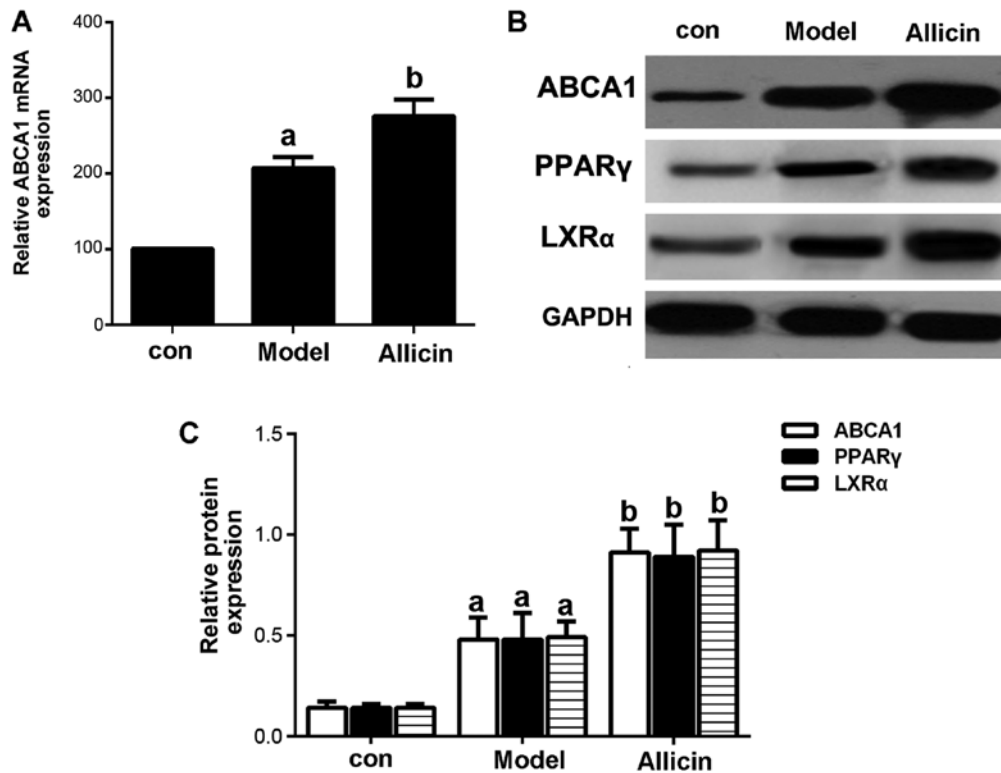


Figure 3. Allicin upregulates the expression of ABCA1 in THP-1 macrophage-derived foam cells. (A) THP-1 macrophage-derived foam cells were stimulated with PMA and ox-LDL (50 mg/l), and were then incubated with 50 μ mol/l ABCA1 siRNA for 6 h, followed by treatment with 5 g/l allicin for 24 h. ABCA1 mRNA expression was analyzed by RT-qPCR. (B and C) THP-1 macrophage-derived foam cells were stimulated with PMA and ox-LDL (50 mg/l), and then treated with 5 g/l allicin for 24 h. The protein expression of ABCA1, LXR α and PPAR γ was determined by western blot analysis. The results are representative of 3 independent experiments. ^aP<0.05 was used to compare the control group; ^bP<0.05 was used to compare the model group. con, control. ABCA1, adenosine triphosphate (ATP)-binding cassette transporters A1; LXR α , liver X receptor α ; PPAR γ , peroxisome proliferator-activated receptor γ ; ox-LDL, oxygenized low density lipoprotein; PMA, phorbol myristate acetate.

Table II. Effect of allicin on cholesterol efflux in THP-1 macrophage-derived foam cells.

Group	Cholesterol efflux (%)
Control	8.92 \pm 1.64
Model group	18.65 \pm 1.75 ^a
Allicin (5 g/l)	43.36 \pm 2.56 ^b

THP-1 macrophages-derived foam cells were treated with 2 μ Ci/ml [³H]cholesterol followed by treatment with allicin at 5 g/l for an additional 24 h. Cells were then incubated with or without 10 mg/l apoA-I for 8 h. [³H]cholesterol radioactivity was measured. ^aP<0.05 compared with control; ^bP<0.05 compared with model group. apoA-I, apolipoprotein A-I. Data are the means \pm SD, n=3. Cholesterol efflux values are in percentages (%).

Discussion

Both clinical and reference studies have reported that allicin may help in delaying the progression of cardiovascular diseases. Atherosclerosis is the basis of cardiovascular diseases; cellular cholesterol accumulates in lipid-engorged macrophage foam cells, thereby driving lipid deposition to the core of atherosclerosis (1). Thus, inhibiting the formation of foam cells is an important therapeutic strategy for atherosclerosis.

ABCA1-mediated cholesterol efflux is a process through which excess cell cholesterol of foam cells is picked up by HDL particles and delivered to the liver for final excretion (3). In the present study, our data supported the finding that allicin upregulates the expression of ABCA1 to increase cholesterol efflux and reduce cellular cholesterol and CE via PPAR γ /LXR α signaling in THP-1 macrophage-derived foam cells.

Allicin is a major active component that can be extracted from garlic samples. It has been demonstrated that allicin exerts anti-inflammatory, antioxidant and lipid-modulating effects (14). It has also been shown that allicin can i) protect vascular endothelial cells by delaying the oxidation of LDL, ii) prevent blood clots by inhibiting platelet aggregation, and iii) increase HDL-C but reduce TC, triglyceride and LDL levels (17). Animal tests have also revealed that allicin reduces the atherosclerotic plaque area of LDLR^{-/-} and apoE^{-/-} mice and significantly reduces the level of cholesterol in mice (18); however, the molecular mechanisms involved are unclear. Subsequent studies have indicated that allicin can regulate the NF- κ B signaling pathway, as well as inhibit the expression of interleukin-6 and tumor necrosis factor- α (17,19). Inflammation and cholesterol metabolism disorder are considered major risk factors that give rise to atherosclerosis; however, allicin can be potentially used as an anti-atherosclerotic. However, studies on the effects exerted by allicin on lipid accumulation in foam cells are relatively few. On the basis of these studies, the current study determined the

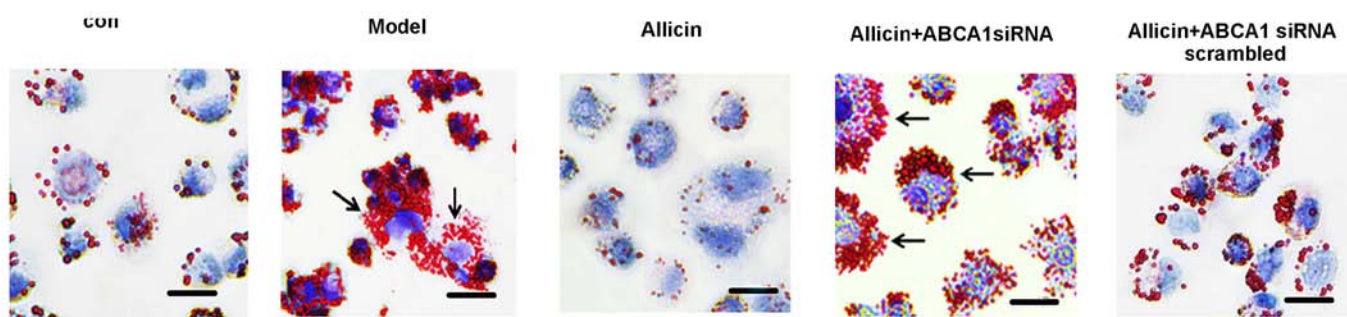


Figure 4. Knockdown ABCA1 attenuates the decreasing effects of allicin on lipid droplet accumulation in THP-1 macrophage-derived foam cells. THP-1 macrophage-derived foam cells were stimulated with PMA and ox-LDL (50 mg/l), and then incubated with 50 μmol/ml ABCA1 siRNA for 6 h, followed by treatment with 5 g/l allicin for 24 h. The cells were then stained with Oil Red O. Scale bar, 15 μm. Magnification, x200. Black arrows indicate foam cells. ABCA1, adenosine triphosphate (ATP)-binding cassette transporters A1; ox-LDL, oxygenized low density lipoprotein; PMA, phorbol myristate acetate.

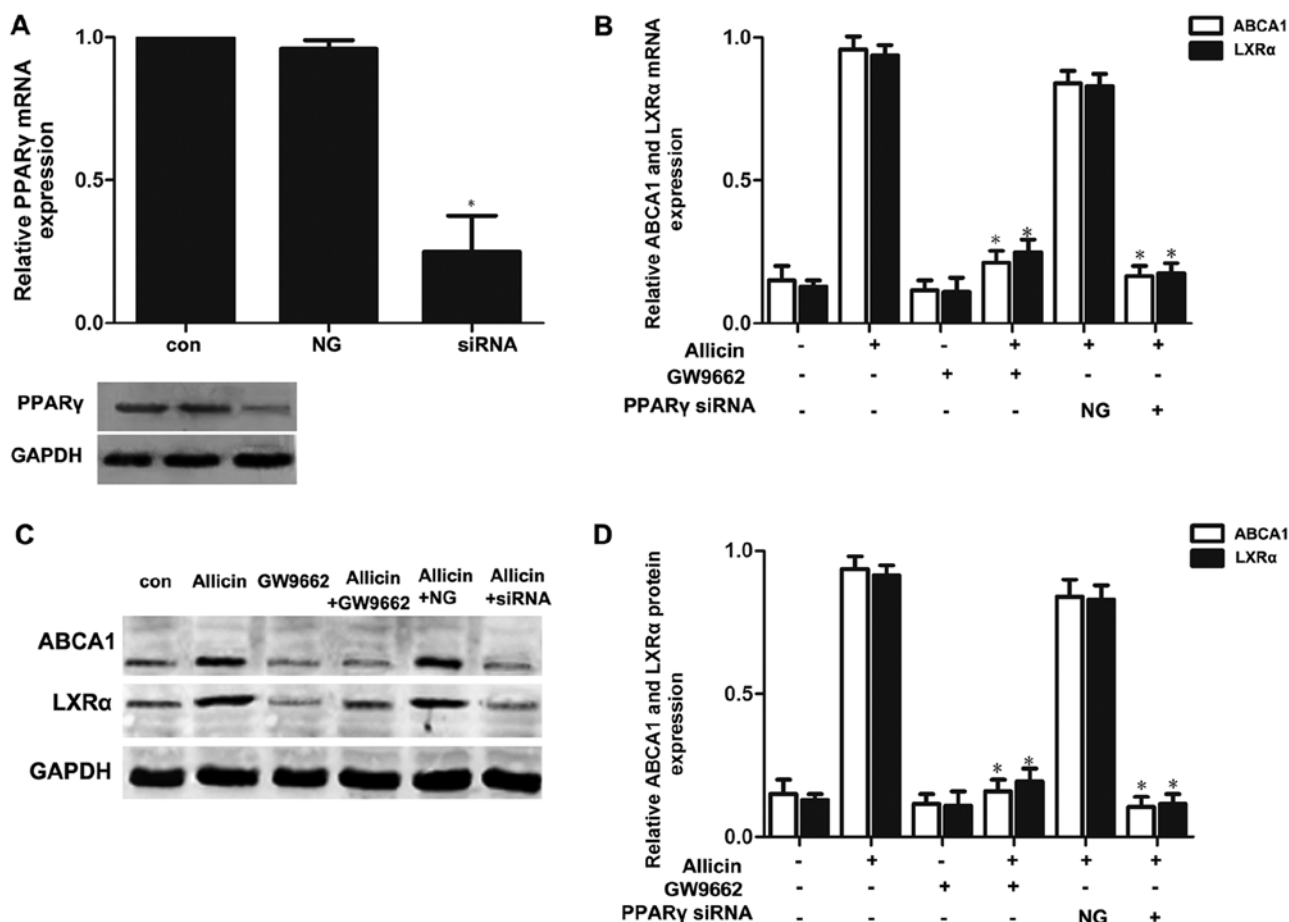


Figure 5. PPAR γ is involved in the upregulation of ABCA1 expression induced by allicin. (A) Small interfering RNA (siRNA) inhibited PPAR γ expression. THP-1 macrophage-derived foam cells were transfected with scrambled or PPAR γ siRNA. PPAR γ expression was determined by western blot analysis and RT-qPCR. The results are expressed as the means \pm SD from 3 independent experiments. * P <0.05 vs. controls. (B-D) Knockdown of PPAR γ attenuated the allicin-induced upregulation of ABCA1 and LXR α expression. THP-macrophage-derived foam cells were transfected with 50 μmol/ml PPAR γ siRNA or 10 mmol/l GW9662 and then incubated with 5 g/l allicin for 24 h. The expression of ABCA1 and LXR α was measured by RT-qPCR and western blot analysis. The results are expressed as the means \pm SD from 3 independent experiments. * P <0.05 vs. allicin group. con, control; NG, negative siRNA; ABCA1, adenosine triphosphate (ATP)-binding cassette transporters A1; LXR α , liver X receptor α .

effects of allicin on cholesterol efflux and lipid accumulation in foam cells and proposed the pertinent mechanism by observing the anti-atherosclerotic effects of allicin from the core process of atherosclerosis occurrence. A new experimental basis was provided for studies regarding the preventive effects of allicin

on atherosclerosis. In addition, this study demonstrated the effects of allicin on the vitality of THP-1 foam cells. According to the results, allicin can degrade cell vitality at higher concentrations. However, cell vitality was not significantly altered at <5 g/l allicin. Moreover, 5 g/l allicin was used to examine the

Table III. Effect of allicin on free cholesterol and cholesterol esters in THP-1 macrophage-derived foam cells

Group	TC	FC	CE	CE/TC (%)
Control	148.32±10.48	97.73±6.29	51.57±5.72	36.48
Model group	543.15±22.36	194.48±21.16	349.23±33.12	63.69
Allicin(5 g/l)	167.68±23.36 ^a	97.18±21.62 ^a	70.82±22.39 ^a	42.23 ^a
Allicin(5 g/l) + ABCA1 scrambled	163.28±23.36	98.38±21.62	64.90±22.39	39.74
Allicin(5 g/l) + ABCA1 siRNA	553.15±23.36 ^b	198.48±27.16 ^b	355.23±31.12 ^b	64.22 ^b

THP-1 macrophage-derived foam cells were divided into 5 groups and cultured at 37°C in media containing 50 μmol/ml ABCA1 siRNA for 6 h, followed by treatment with 5 g/l allicin for 24 h. Cellular cholesterol and cholesterol ester were extracted as described in the Materials and methods. HPLC was performed to determine the levels of cellular TC, FC and CE. Results are expressed as the means ± SD of 3 independent experiments performed in triplicate. ^aP<0.05 vs. control; ^bP<0.05 vs. allicin. TC, total cholesterol; FC, free cholesterol; CE, cholesterol ester; HPLC, high-performance liquid chromatography. Cholesterol levels are in mg/g.

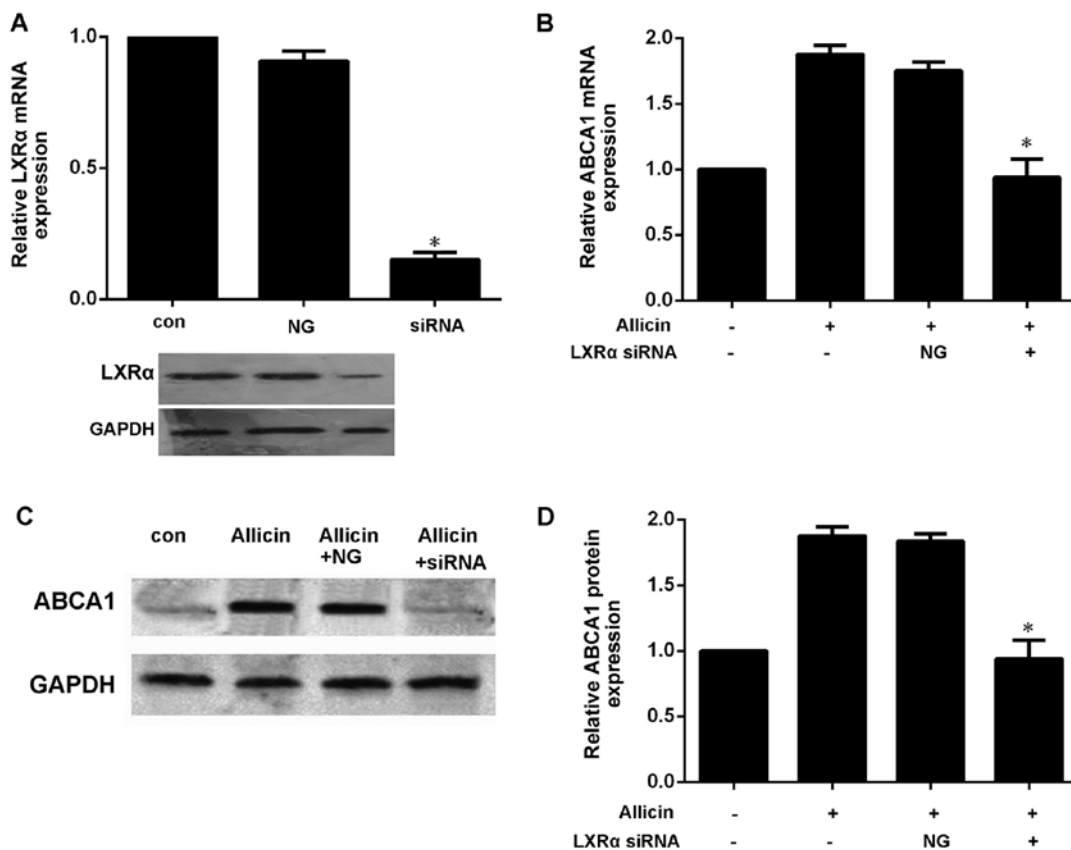


Figure 6. LXRα is involved in the upregulation of ABCA1 expression induced by allicin. (A) Small interfering RNA (siRNA) inhibited the expression of LXRα. THP-1 macrophage-derived foam cells were transfected with scrambled or 50 μmol/ml LXRα siRNA. LXRα expression was determined by western blot analysis and RT-qPCR. The results are expressed as the means ± SD from 3 independent experiments. *P<0.05 vs. controls. (B-D) Knockdown of PPARγ attenuated the allicin-induced upregulation of ABCA1. THP-macrophage-derived foam cells were transfected with 50 μmol/ml LXRα siRNA and then incubated with 5 g/l allicin for 24 h. The expression of ABCA1 and LXRα was measured by RT-qPCR and western blot analysis. The results are expressed as the means ± SD from 3 independent experiments. *P<0.05 vs. allicin group. con, control; NG, negative siRNA; ABCA1, adenosine triphosphate (ATP)-binding cassette transporters A1; LXRα, liver X receptor α.

effects of various treatments durations, and cell vitality was found to be unaltered with time. Finally, 5 g/l allicin was used to process foam cells and observe any change in lipid droplet accumulation in these cells. As shown in Fig. 2 and Table I, 5 g/l allicin decreased lipid accumulation in foam cells and inhibited lipid droplet formation. Thus, allicin exerted a direct inhibitory effect on lipid accumulation in foam cells.

Cholesterol efflux is the main pathway for reducing lipid accumulation in foam cells. ABCA1 is the core protein for regulating RCT. By inducing cholesterol efflux from cells, allicin significantly balanced the lipids in cells. Studies have indicated that ABCA1 is the key protein for driving cholesterol efflux and reducing lipid accumulation in foam cells. Xu *et al* (20) and Liu *et al* (21) reported that the upregulated expression of

ABCA1 significantly facilitated the efflux of cholesterol from THP-1-derived foam cells; it also reduce the levels of TC, FC and CE within foam cells, and reduced lipid accumulation in foam cells. By contrast, the inhibition of ABCA1 expression may also hinder cholesterol efflux and facilitate lipid accumulation in foam cells. Westerterp *et al* (22) and He *et al* (23) also confirmed this observation. On the basis of previous results, we deduced that ABCA1 is the key protein for reducing lipid accumulation in foam cells through the effects of allicin. To prove the correctness of this deduction, we observed the change in ABCA1 expression along with cholesterol efflux from foam cells after processing these cells with 5 g/l allicin. The results indicated that allicin indeed upregulated ABCA1 expression in foam cells and facilitated cholesterol efflux. Subsequently, the cells were transected with ABCA1 siRNA. Allicin was found to facilitate cholesterol efflux from the foam cells, and this reducing effect on lipid accumulation in foam cells was reversed by ABCA1 siRNA. These observations confirmed our deduction and indicated that the upregulation of ABCA1 facilitates cholesterol efflux and decreases lipid accumulation in foam cells through allicin treatment.

The PPAR γ /LXR α pathway is the core mechanism for regulating ABCA1 expression, and the effect of this pathway on ABCA1 expression has been widely accepted (24). Liver X receptor, as a nuclear transcription factor, can regulate multiple genes in the cholesterol-transporting pathway, e.g., transcriptional regulation of ABCA1 and ABCG1 (25). Another study demonstrated that the LXR stimulant, T0901317, inhibited the progression of atherosclerosis in mice (26). Cellular experiments have confirmed that T0901317 upregulates ABCA1 and ABCG1 expression by activating LXR α in macrophages, thereby driving cholesterol within the cells to flow to apoAI and HDL and inhibiting the formation of foam cells (27). However, another study indicated that LXR α expression was regulated by other nuclear transcription factors, e.g., PPAR (28). PPAR, a type of nuclear transcription factor, has 3 subtypes: PPAR α , PPAR β and PPAR γ . These nuclear transcription factors are combined with their respective ligands to alter spatial conformation and subsequently combine with the PPAR response element within the target gene promoter to regulate the transcription of the target gene. These nuclear transcription factors can also activate LXR α and combine with AX receptor to form a heterodimer, which regulates the transcription of the target gene. Among the 3 PPAR subtypes, PPAR γ can regulate glucose and lipid metabolism, as well as inflammatory response and immunity (29,30). Hence, these transcription factors are the key to the transcriptional regulation of cell differentiation and lipid metabolism. It has been demonstrated that PPAR γ upregulates LXR α expression (31) and that allicin upregulates PPAR γ expression and regulates the inflammatory response of endothelial cells (17). Therefore, the PPAR γ /LXR α pathway contributes to the promoting effect of allicin on ABCA1 expression. As shown in Fig. 3, that allicin upregulated PPAR γ and LXR α protein expression in THP-1 foam cells. Furthermore, to determine the participation of the PPAR γ /LXR α pathway in the upregulation of ABCA1 expression by allicin, we transfected the THP-1 foam cells with PPAR γ siRNA or an inhibitor, along with LXR α siRNA. Subsequently, we observed any change in ABCA1 expression. The results indicated that following transfection with PPAR γ siRNA or an inhibitor

along with LXR α siRNA, the upregulatory effect of allicin on ABCA1 expression was significantly reversed. Therefore, allicin upregulates ABCA1 expression, facilitates cholesterol efflux, and reduces lipid accumulation in THP-1 foam cells by activating the PPAR γ /LXR α pathway.

In conclusion, this study proves that allicin reduces lipid accumulation through the upregulation of ABCA1 expression via PPAR γ /LXR α signaling in THP-1 macrophage-derived foam cells. Treatment with allicin in the field of cardiovascular disease and the use of arsenic drug research and development may provide a novel strategy for the prevention and/or treatment of atherosclerosis. These findings offer a new perspective on the use of allicin for the treatment of atherosclerosis.

Acknowledgements

The present study was supported by grants from the Medical Foundation of Huizhou (no. 2015Y134); the Medical Research Foundation of Guangdong (no. A2015620); the Graduate Student Research Innovation Project of Hunan (no. CX2013B396); and the Natural Science Foundation of China (grant no. 81600342).

References

- Zeller I and Srivastava S: Macrophage functions in atherosclerosis. *Circ Res* 115: e83-e85, 2014.
- Yu XH, Fu YC, Zhang DW, Yin K and Tang CK: Foam cells in atherosclerosis. *Clin Chim Acta* 424: 245-252, 2013.
- Westerterp M, Bochem AE, Yvan-Charvet L, Murphy AJ, Wang N and Tall AR: ATP-binding cassette transporters, atherosclerosis, and inflammation. *Circ Res* 114: 157-170, 2014.
- Heinecke JW: Small HDL promotes cholesterol efflux by the ABCA1 pathway in macrophages: Implications for therapies targeted to HDL. *Circ Res* 116: 1101-1103, 2015.
- Negi SI, Brautbar A, Virani SS, Anand A, Polisecki E, Asztalos BF, Ballantyne CM, Schaefer EJ and Jones PH: A novel mutation in the ABCA1 gene causing an atypical phenotype of Tangier disease. *J Clin Lipidol* 7: 82-87, 2013.
- Pervaiz MA, Gau G, Jaffe AS, Saenger AK, Baudhuin L and Ellison J: A Non-classical presentation of tangier disease with three ABCA1 mutations. *JIMD Rep* 4: 109-111, 2012.
- Fitzgerald ML, Mujawar Z and Tamehiro N: ABC transporters, atherosclerosis and inflammation. *Atherosclerosis* 211: 361-370, 2010.
- Huang CX and Zhang YL: The target of regulating the ATP-binding cassette A1 protein (ABCA1): Promoting ABCA1-mediated cholesterol efflux in different cells. *Curr Pharm Biotechnol* 14: 623-631, 2013.
- Chinetti G, Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, Teissier E, Minnich A, Jaye M, Duverger N, *et al*: PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat Med* 7: 53-58, 2001.
- Schmitz G and Langmann T: Transcriptional regulatory networks in lipid metabolism control ABCA1 expression. *Biochim Biophys Acta* 1735: 1-19, 2005.
- Borlinghaus J, Albrecht F, Gruhlke MC, Nwachukwu ID and Slusarenko AJ: Allicin: Chemistry and biological properties. *Molecules* 19: 12591-12618, 2014.
- Chen S, Tang Y, Qian Y, Chen R, Zhang L, Wo L and Chai H: Allicin prevents H₂O₂-induced apoptosis of HUVECs by inhibiting an oxidative stress pathway. *BMC Complement Altern Med* 14: 321-329, 2014.
- Sung J, Harfouche Y, De La Cruz M, Zamora MP, Liu Y, Rego JA and Buckley NE: Garlic (*Allium sativum*) stimulates lipopolysaccharide-induced tumor necrosis factor-alpha production from J774A.1 murine macrophages. *Phytother Res* 29: 288-294, 2015.
- Gonen A, Harats D, Rabinkov A, Miron T, Mirelman D, Wilchek M, Weiner L, Ulman E, Levkovitz H, Ben-Shushan D, *et al*: The antiatherogenic effect of allicin: Possible mode of action. *Pathobiology* 72: 325-334, 2005.

15. Lin XL, Liu MH, Hu HJ, Feng HR, Fan XJ, Zou WW, Pan YQ, Hu XM and Wang Z: Curcumin enhanced cholesterol efflux by upregulating ABCA1 expression through AMPK-SIRT1-LXR α signaling in THP-1 macrophage-derived foam cells. *DNA Cell Biol* 34: 561-572, 2015.
16. Jin X, Freeman SR, Vaisman B, Liu Y, Chang J, Varsano N, Addadi L, Remaley A and Kruth HS: ABCA1 contributes to macrophage deposition of extracellular cholesterol. *J Lipid Res* 56: 1720-1726, 2015.
17. Mo SJ, Son EW, Rhee DK and Pyo S: Modulation of TNF- α -induced ICAM-1 expression, NO and H₂O₂ production by alginate, allicin and ascorbic acid in human endothelial cells. *Arch Pharm Res* 26: 244-251, 2003.
18. Li RK, Li JF, Zhou XR, Yin M, Zhang L and Pan J: Effects of allicin on plasma lipid metabolism of atherosclerotic mice. *Chin J Clin (Electronic Version)* 1: 29-33, 2007.
19. Li C, Lun W, Zhao X, Lei S, Guo Y, Ma J and Zhi F: Allicin alleviates inflammation of trinitrobenzenesulfonic acid-induced rats and suppresses p38 and JNK pathways in Caco-2 cells. *Mediators Inflamm* 2015: 434692, 2015.
20. Xu X, Li Q, Pang L, Huang G, Huang J, Shi M, Sun X and Wang Y: Arctigenin promotes cholesterol efflux from THP-1 macrophages through PPAR- γ /LXR- α signaling pathway. *Biochem Biophys Res Commun* 441: 321-326, 2013.
21. Liu XY, Lu Q, Ouyang XP, Tang SL, Zhao GJ, Lv YC, He PP, Kuang HJ, Tang YY, Fu Y, *et al*: Apelin-13 increases expression of ATP-binding cassette transporter A1 via activating protein kinase C α signaling in THP-1 macrophage-derived foam cells. *Atherosclerosis* 226: 398-407, 2013.
22. Westerterp M, Murphy AJ, Wang M, Pagler TA, Vengrenyuk Y, Kappus MS, Gorman DJ, Nagareddy PR, Zhu X, Abramowicz S, *et al*: Deficiency of ATP-binding cassette transporters A1 and G1 in macrophages increases inflammation and accelerates atherosclerosis in mice. *Circ Res* 112: 1456-1465, 2013.
23. He Y, Zhang L, Li Z, Gao H, Yue Z, Liu Z, Liu X, Feng X and Liu P: RIP140 triggers foam-cell formation by repressing ABCA1/G1 expression and cholesterol efflux via liver X receptor. *FEBS Lett* 589: 455-460, 2015.
24. Chinetti-Gbaguidi G, Baron M, Bouhlef MA, Vanhoutte J, Copin C, Sebti Y, Derudas B, Mayi T, Bories G, Tailleux A, *et al*: Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPAR γ and LXR α pathways. *Circ Res* 108: 985-995, 2011.
25. Chen SG, Xiao J, Liu XH, Liu MM, Mo ZC, Yin K, Zhao GJ, Jiang J, Cui LB, Tan CZ, *et al*: Ibrolipim increases ABCA1/G1 expression by the LXR α signaling pathway in THP-1 macrophage-derived foam cells. *Acta Pharmacol Sin* 31: 1343-1349, 2010.
26. Kappus MS, Murphy AJ, Abramowicz S, Ntonga V, Welch CL, Tall AR and Westerterp M: Activation of liver X receptor decreases atherosclerosis in *Ldlr*^{-/-} mice in the absence of ATP-binding cassette transporters A1 and G1 in myeloid cells. *Arterioscler Thromb Vasc Biol* 34: 279-284, 2014.
27. Ma AZ, Song ZY and Zhang Q: Cholesterol efflux is LXR α isoform-dependent in human macrophages. *BMC Cardiovasc Disord* 14: 80, 2014.
28. Parikh M, Patel K, Soni S and Gandhi T: Liver X receptor: A cardinal target for atherosclerosis and beyond. *J Atheroscler Thromb* 21: 519-531, 2014.
29. Monsalve FA, Pyarasani RD, Delgado-Lopez F and Moore-Carrasco R: Peroxisome proliferator-activated receptor targets for the treatment of metabolic diseases. *Mediators Inflamm* 2013: 549627, 2013.
30. Cheng X, Liu X, Song L, He Y, Li X and Zhang H: Atorvastatin inhibits macrophage-derived foam cell formation by suppressing the activation of PPAR γ and NF- κ B pathway. *Nan Fang Yi Ke Da Xue Xue Bao* 34: 896-900, 2014 (In Chinese).
31. Baranowski M, Blachnio-Zabielska AU, Zabielski P, Harasim E, Harasiuk D, Chabowski A and Gorski J: Liver X receptor agonist T0901317 enhanced peroxisome proliferator-activated receptor- δ expression and fatty acid oxidation in rat skeletal muscle. *J Physiol Pharmacol* 64: 289-297, 2013.