



# Hesperetin inhibits foam cell formation and promotes cholesterol efflux in THP-1-derived macrophages by activating LXR $\alpha$ signal in an AMPK-dependent manner

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## Abstract

Cholesterol efflux from macrophages is the first step of reverse cholesterol transport (RCT), whose increase inhibits cholesterol accumulation and foam cell formation to suppress atherogenesis. Hesperetin has been reported to exert several protective effects on cardiovascular diseases, while little is known about the role of hesperetin and its underlying mechanism in macrophage foam cell formation. In this study, we sought to investigate the potential effects of hesperetin on foam cell formation and cholesterol efflux by using human macrophages, focusing on liver X receptor alpha (LXR $\alpha$ ) and AMPK. We found that hesperetin treatment reduced foam cell formation, intracellular cholesterol levels and the cholesterol esterification rate, and increased cholesterol efflux in THP-1 macrophages. Hesperetin increased the levels of LXR $\alpha$  protein and its targets, including ABCA1, ABCG1, SR-BI, and phosphorylated-AMPK. Meanwhile, the hesperetin-induced increase in LXR $\alpha$  expression was further increased by the AMPK agonist and inhibited by an AMPK inhibitor. Meanwhile, hesperetin increased the levels of LXR $\alpha$  mRNA and its target genes, all of which were decreased in cells transfected with the AMPK $\alpha$ 1/ $\alpha$ 2 small interfering RNA (siRNA). Furthermore, the hesperetin-induced inhibition of foam cell formation and promotion of cholesterol efflux were decreased by transfection of AMPK $\alpha$ 1/ $\alpha$ 2 siRNA. In conclusions, We are the first to report that hesperetin activate AMPK in THP-1-derived macrophages. This activation upregulates LXR $\alpha$  and its targets, including ABCA1, ABCG1 and SR-BI, which significantly inhibits foam cell formation and promotes cholesterol efflux. Our results highlight the therapeutic potential of hesperetin to possibly reduce foam cell formation. This new mechanism might contribute the anti-atherogenic effects of hesperetin.

**Keywords** Reverse cholesterol transport · Macrophage · Hesperetin · Lxr $\alpha$  · AMPK

## Introduction

Reverse cholesterol transport (RCT) is an effective approach to alleviate hypercholesterolemia and atherosclerosis [4]. It refers to the process by which excess cholesterol from

lipid-laden peripheral cells is transferred to plasma high-density lipoprotein, then transported to the liver for bile acid synthesis, and finally excreted in the feces.

Liver X receptors (LXRs) are ligand-activated nuclear transcription factors comprising two isoforms, LXR $\alpha$  and LXR $\beta$ . LXR $\alpha$  is predominantly expressed in tissues and cells related to lipid metabolism, such as the liver, intestine, adipose tissue and macrophages [20]. LXR $\alpha$  plays a pivotal role in the transcriptional regulation of cholesterol homeostasis by regulating the transcription of genes that encode proteins involved in cholesterol biosynthesis, catabolism, secretion and reverse transport. Cellular cholesterol efflux, the initial step of RCT, is primarily mediated by three identified target proteins of LXR $\alpha$ , namely, ATP-binding cassette subfamily A member 1 (ABCA1), ATP-binding cassette subfamily G member 1 (ABCG1) and scavenger receptor class B type I (SR-BI) [29]. Activation of the LXR $\alpha$  signaling pathway by endogenous lipid ligands or synthetic agonists such as T0901317 promotes

## Highlights

1. Hesperetin inhibits foam cell formation and promotes cholesterol efflux in THP-1-derived macrophages.
2. Hesperetin upregulates the expression of LXR $\alpha$  protein and mRNA in THP-1-derived macrophages.
3. Hesperetin increases the expression of LXR $\alpha$  target proteins including ABCA1, ABCG1, and SR-BI.
4. AMPK is implicated in hesperetin-induced activation of LXR $\alpha$  signal and LXR $\alpha$  signal-mediated inhibition of foam cell formation and promotion of cholesterol efflux.

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cellular cholesterol efflux from lipid-laden macrophages to attenuate atherosclerosis progression [15].

The transcriptional activity of LXRs, similar to other nuclear receptors, is regulated by posttranslational modifications, including acetylation, SUMOylation, and phosphorylation [16]. Previous studies have linked LXR phosphorylation to some kinases, such as mitogen-activated protein kinases, protein kinase C, casein kinase, and adenosine monophosphate-activated protein kinase (AMPK) [5, 26, 28]. AMPK is a ubiquitously expressed serine/threonine protein kinase complex that is responsible for maintaining balance between anabolic and catabolic pathways for cellular energy homeostasis. Once activated, AMPK triggers the catalytic process that generates ATP, which controls the biosynthesis and consumption of ATP [12]. Based on considerable evidence, AMPK activation inhibits the formation of foam cells and the deposition of atherosclerotic plaques [7, 16]. However, the association between AMPK and LXR $\alpha$  in a cellular model of atherosclerosis remains to be illustrated.

Flavonoid compounds possess multiple biological and pharmacological activities [3, 27]. Hesperetin, a citrus bioflavonoid, possesses prominent anti-inflammatory, anti-oxidant, anti-apoptosis, insulin-sensitizing and lipid-lowering activities, and exerts protective effects on the cardiovascular system, nervous system and the homeostasis of lipid and glucose metabolism [6, 8, 13]. Hesperetin reduces lipid accumulation in 3T3-L1 cells and rats liver [1, 14]. However, the effects of hesperetin on lipid accumulation in macrophages and the underlying mechanism remain largely obscure. Recently, hesperetin has been suggested as an enhancer of cholesterol efflux and a potent bioactivator of LXR $\alpha$  and AMPK [9, 24]. Based on these observations, we hypothesized that the enhancement of RCT by hesperetin inhibits foam cell formation and the LXR $\alpha$  and AMPK signaling pathway may be involved in this process. Therefore, we examined the effects of hesperetin on foam cell formation, cholesterol levels and cholesterol efflux in THP-1 macrophages. Furthermore, we investigated the underlying mechanisms regulating the levels of the LXR $\alpha$  mRNA and protein, as well as its downstream targets, and the role of AMPK in this process.

## Materials and methods

### Materials

RPMI-1640 medium, fetal bovine serum (FBS), and the BCA assay kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Phorbol 12-myristate 13-acetate (PMA), T0901317 (293,754–55-9), Compound C (866,405–64-3), AICA-riboside (3031–94-5), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium

bromide (MTT) (298–93-1), Oil Red O (1320–06-5), and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Cholesterol Efflux Assay Kit (ab196985), anti-LXR alpha antibody (ab176323), anti-ABCA1 antibody (ab18180), anti-ABCG1 antibody (ab52617), and anti-scavenging receptor SR-BI antibody (ab217318) were purchased from Abcam (Cambridge, MA, USA). RIPA lysis buffer, the anti-phospho-AMPK $\alpha$  antibody (50,081), anti- $\beta$ -actin antibody (4970), HRP-conjugated anti-rabbit IgG antibody (7074), and HRP-conjugated anti-mouse IgG antibody (7076) were purchased from Cell Signaling Technology (Danvers, MA, USA). Polyvinylidene difluoride membranes and the ECL kit (WBKLS0500) were purchased from Merck Millipore (Billerica, MA, USA). The control siRNA (sc-37007), AMPK alpha 1 siRNA (sc-29673), and AMPK alpha 2 siRNA (sc-38923) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The Total RNA Extraction Reagent (R401-01), HiScript $\text{®}$  II Q RT Super Mix (Q711-02/03), and ChamQ Universal SYBR qPCR Master Mix (Q711-02) were purchased from Vazyme Biotech Ltd. (Nanjing, China). The tissue total cholesterol assay kit (E1015) and tissue free cholesterol assay kit (E1016) were purchased from Applygen Technologies Inc. (Beijing, China). Oxidized low-density lipoprotein (ox-LDL), hesperetin (520–33-2), phosphatase inhibitor cocktail, and anti-AMPK alpha antibody (66,536–1-Ig) were purchased from Yiyuan Biotechnologies (Guangdong, China), Chengdu Chroma-Biotechnology Ltd. (Sichuan, China), Roche (Basel, Switzerland), and Proteintech Group Inc. (Rosemont, USA), respectively.

### Cell culture and treatments

The THP-1 human monocytic leukemia cell line (SCSP-567) was obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). THP-1 cells were grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 2.5  $\mu$ g/mL amphotericin B and 2 mM L-glutamine in a humidified atmosphere containing 5% CO $_2$  at 37  $^{\circ}$ C. Cells ( $1 \times 10^6$  cells/mL) were cultured with 160 nM phorbol12-myristate 13-acetate for 24 h to facilitate their differentiation into macrophages. THP-1-derived macrophages were then incubated with 80  $\mu$ g/mL ox-LDL combined with different compounds for another 24 h.

Reagents were prepared in a minimal volume of dimethyl sulfoxide (DMSO) and stored at -20  $^{\circ}$ C. For treatments, hesperetin (10–1000  $\mu$ mol/L), T0901317 (10  $\mu$ mol/L), AICAR (100  $\mu$ mol/L) and Compound C (10  $\mu$ mol/L) were added to the culture medium. The concentrations of reagents were determined considering the previous reports [30, 32].

## Cell viability

THP-1 cells were seeded in 96-well plates at a density of  $1 \times 10^4$  cells per well and cultured overnight. The cells were then treated with gradient dilutions of hesperetin (0–1000  $\mu\text{mol/L}$ ) for 24 h. Next, 5 mg/mL MTT in RPMI-1640 medium was added to each well of the plate and incubated with the cells at 37 °C for 4 h. Subsequently, the medium was discarded and cells were incubated with DMSO for 10 min at room temperature. The absorbance was measured at 570 nm with a microplate reader (BioTek Instruments Inc., Winooski, USA).

## Oil red O staining and analysis of lipid accumulation

THP-1 cells were seeded in 6-well plates at a density of  $2 \times 10^5$  cells per well and treated as described above. Subsequently, THP-1-derived macrophage foam cells were rinsed 3 times with cold phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde for 30 min. After 3 rinses with distilled  $\text{H}_2\text{O}$ , the cells were stained with 0.5 mL of a freshly filtered Oil Red O staining solution (0.5% Oil red O in 60% isopropanol) per well for 30 min at room temperature. Foam cells were observed and photographed using a light microscope (OLYMPUS BX53, Olympus, Tokyo, Japan).

After Oil Red O staining, intracellularly stained lipid droplets were extracted with isopropanol. The absorbance was measured at 492 nm with a microplate reader (BioTek Instruments Inc., Winooski, USA). The quantitative results were corrected after parallel experiments to determine cellular protein contents.

## Analysis of cellular cholesterol accumulation and cholesterol efflux

The cellular TC and FC levels were determined using the tissue total cholesterol assay kit and tissue free cholesterol assay kit. All experiments were performed according to the manufacturer's instructions. The contents of cholesteryl ester (CE) were determined by subtracting the level of FC from the total cholesterol concentration. Cellular protein contents were calculated using a BCA assay kit.

The macrophage cholesterol efflux capacity was determined using a Cholesterol Efflux Assay Kit. The procedure was performed according to the manufacturer's instructions. Briefly, THP-1 macrophages were pretreated with fluorescently labeled cholesterol for 6 h and then treated with or without hesperetin for 24 h. The cell supernatant was collected and cells were solubilized with cell lysis buffer. A microplate reader was used to analyze the absorbance of

the supernatant and lysates at 490 nm. The percentage of cholesterol efflux was calculated as the ratio between the fluorescence intensity of the media and fluorescence intensity of the cell lysate plus media  $\times 100\%$ .

## Western blot analysis

Cells in 6-well plates were lysed with RIPA lysis buffer containing 1 mM PMSF and 1% of a phosphatase inhibitor cocktail (PhosSTOP, Roche group, Swiss). The supernatant was collected by centrifugation at 14,000 rpm for 15 min at 4 °C. The concentration of the cell lysate was determined using a Pierce BCA Protein Assay Kit. Equal amounts of proteins (60  $\mu\text{g}$  each) were separated using 10% sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes at 300 mA for 1.5 h at 4 °C. Membranes were incubated with primary antibodies overnight at 4 °C after 1 h of blocking in 5% skim milk (Becton, Dickinson and Company, USA) at room temperature. Membranes were then washed with TBST for a total of 15 min and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were detected using an ECL kit, and band intensity was analyzed using Image Lab 5. 2. 1 software (Bio-Rad, Hercules, USA).

Primary antibodies included the anti-AMPK alpha antibody, anti-LXR alpha antibody, anti-ABCA1 antibody, anti-ABCG1 antibody, anti-scavenging receptor SR-BI antibody, and anti- $\beta$ -actin antibody. Secondary antibodies included the HRP-conjugated anti-rabbit IgG antibody and HRP-conjugated anti-mouse IgG antibody.

## Transfection of small interfering RNAs

THP-1 macrophages were transfected with a nonsilencing control siRNA or siRNA targeting AMPK $\alpha 1/\alpha 2$  according to the manufacturer's instructions. Briefly, THP-1-derived macrophages were seeded into 6-well plates at a density of  $2 \times 10^5$  cells/well. The siRNA duplex solution was added to the dilute transfection reagent and incubated for 30 min at room temperature. After washing the cells once with transfection medium, cells were incubated with the transfection reagent mixture in transfection medium (siRNA final concentration at 60 nM) at 37 °C for 6 h. Then, 1 mL of RPMI-1640 medium containing 2 times the normal concentrations of serum and antibiotics was added to each wells and incubated with the cells for another 24 h. Subsequently, cells were treated with different reagents for 24 h. Target gene knockdown was validated using real time RT-PCR, and GADPH expression served as an internal control.

## Real-time RT-PCR analysis

THP-1 macrophages were seeded in 6-well plates. After transfection and treatments as described above, total mRNA was extracted using the Total RNA Extraction Reagent. First-strand cDNAs were synthesized from 1 µg of total RNA using a HiScript® II Q RT Super Mix. Then, 2 µL of cDNAs were amplified using the ChamQ Universal SYBR qPCR Master Mix in a total volume of 25 µL. Real-time quantitative polymerase chain reaction was conducted with a Bio-Rad CFX96 cycler and specific thermocycling conditions according to the manufacturer's instructions. GAPDH served as the internal control and quantitative measurements were analyzed using the  $\Delta\Delta\text{Ct}$  method. The primer sequences are presented in Table 1.

## Statistical analysis

Statistical analysis was carried out with the help of Prism 7.0 software (Graphpad Software, CA, USA).

*P* values were calculated using ANOVA followed by Bonferroni and Tamhane's T2 post-hoc analysis. Statistical significance was set at  $P < 0.05$  (Fig. 1).

## Results

### Hesperetin inhibits foam cell formation and reduces lipid accumulation in THP-1 macrophages

First, we observed the effects of different concentrations (0–1000 µmol/L) of hesperetin on THP-1 macrophage activity. A high concentration of hesperetin decreased the viability of THP-1 macrophages, but a maximum hesperetin

concentration of 100 µM did not affect cell viability (Fig. 2a). Therefore, we chose 10, 50 and 100 µmol/L concentrations of hesperetin to conduct the next experiments.

THP-1 macrophages were incubated with 80 µg/mL ox-LDL in the presence or absence of hesperetin for 24 h to investigate the effect of hesperetin on foam cell formation and lipid accumulation. Then, the Oil Red O-stained lipid droplets in cells were observed. Hesperetin significantly reduced the size and number of lipid droplets in THP-1 macrophage foam cells (Fig. 2c). A quantitative analysis of intracellular lipid contents was performed after the extraction of intracellular dye in isopropanol. Hesperetin reduced the lipid contents in THP-1 macrophage foam cells (Fig. 2b).

### Hesperetin reduces cholesterol accumulation and promotes cholesterol efflux in THP-1 macrophage foam cells.

Since hesperetin exerted a significant effect on reducing lipid accumulation, we postulated that hesperetin would also inhibit cholesterol accumulation. As shown in Fig. 3a and b, compared with control cells, total cholesterol (TC) levels, cholesterol ester (CE) levels, and the esterification rate (CE/TC) were significantly increased after treatment with ox-LDL. However, hesperetin reduced intracellular TC and CE levels, as well as the esterification rate.

Furthermore, we assessed cholesterol efflux to investigate how hesperetin reduces the intracellular cholesterol level. As shown in Fig. 3c, hesperetin increased cholesterol efflux to 11.31%, 15.38% and 18.58% at concentrations of 10 µmol/L, 50 µmol/L, and 100 µmol/L, respectively, all which were significantly different from cells treated with ox-LDL alone.

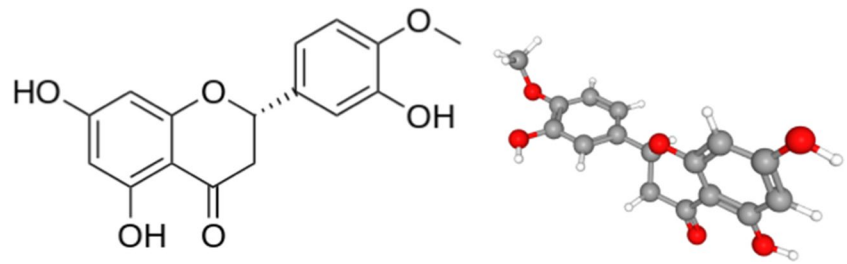
### Hesperetin upregulates the expression of the LXR $\alpha$ protein and mRNA and LXR $\alpha$ target proteins in THP-1 macrophages.

LXR $\alpha$  signaling plays a critical role in cellular cholesterol homeostasis. Therefore, we assessed the effects of hesperetin on levels of the LXR $\alpha$  protein and mRNA. As shown in Fig. 4a-d, hesperetin increased the levels of the LXR $\alpha$  protein and mRNA, while this increase was lower than in cells treated with T0901317. We chose the best effective concentration of hesperetin at 100 µM to further validate its role in activating LXR $\alpha$ . Interestingly, when cells were treated with hesperetin and T0901317, a accumulative effects on the levels of the LXR $\alpha$  protein and mRNA was observed, which was a significantly different compared to cells treated with T0901317 alone.

We further examined the role of hesperetin in modulating the levels of LXR $\alpha$  downstream target proteins, including ABCA1, ABCG1, and SR-BI. Hesperetin increased the

**Table 1** Sequences of primer for real-time RT-PCR

Gene	Primer
GAPDH	F 5'-CAACGTGTCAGTGGTGGACCTG-3' R 5'-GTGTCGCTGTTGAAGTCAGAGGAG-3'
ABCA1	F 5'-CCCTGTGGAATGTACCTATGTG-3' R 5'-GAGGTGTCCCAAAGATGCAA-3'
LXR $\alpha$	F 5'-GGATTTGGACAGTGCCTTGGT-3' R 5'-GTCAGGAGGAATGTCAGGCAC-3'
ABCG1	F 5'-CAGTCGCTCCTTAGCACCA-3' R 5'-TCCATGCTCGGACTCTCTG-3'
SRBI	F 5'-TTGCCAACGGGTCCATCTAC-3' R 5'-GAAACAAGGGGCACTGAAC-3'
AMPK $\alpha$ 1	F 5'-TGTAAGAATGGAAGGCTGGATGA-3' R 5'-GGACCACATATGCCTGTGA-3'
AMPK $\alpha$ 2	F 5'-GGTGATCAGCACTCCAACAGA-3' R 5'-TCTCTTCAACCCGTCCATGC-3'

**Fig. 1** Structure of hesperetin

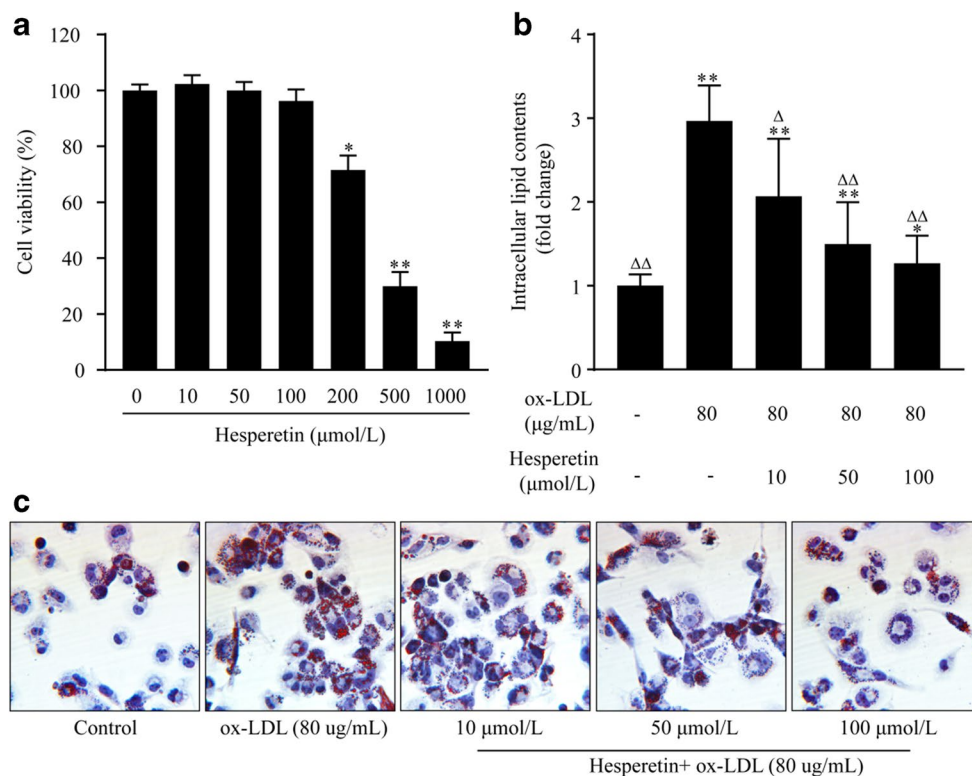
levels of the ABCA1, ABCG1 and SR-BI proteins. Among these proteins, ABCG1 levels were increased by hesperetin to a slightly greater extent than by T0901317. However, the increases in the levels of ABCA1 and SR-BI were lower than the increases induced by T0901317 (Fig. 4c-g).

### AMPK is implicated in hesperetin-induced LXR $\alpha$ activation.

AMPK has been described as a key regulator of lipid synthesis. Therefore, the effect of hesperetin on AMPK activation was examined. As shown in Fig. 5a, hesperetin significantly

increased the level of the phosphorylated AMPK (pAMPK) protein by 1.5-fold compared with the control group of THP-1 macrophages. Meanwhile, an accumulative effect was observed when cells were treated with the combination of AICAR and hesperetin. In cells administered this treatment, the level of the pAMPK protein are significantly increased 1.5-fold compared with the cells treated with AICAR alone. However, a statistically significant difference in total levels of the AMPK protein was not observed between groups (Fig. 5b).

LXR $\alpha$  has been reported to be regulated by the phosphorylation of AMPK. We therefore investigated whether the activation of AMPK induced by hesperetin effectively induces LXR $\alpha$

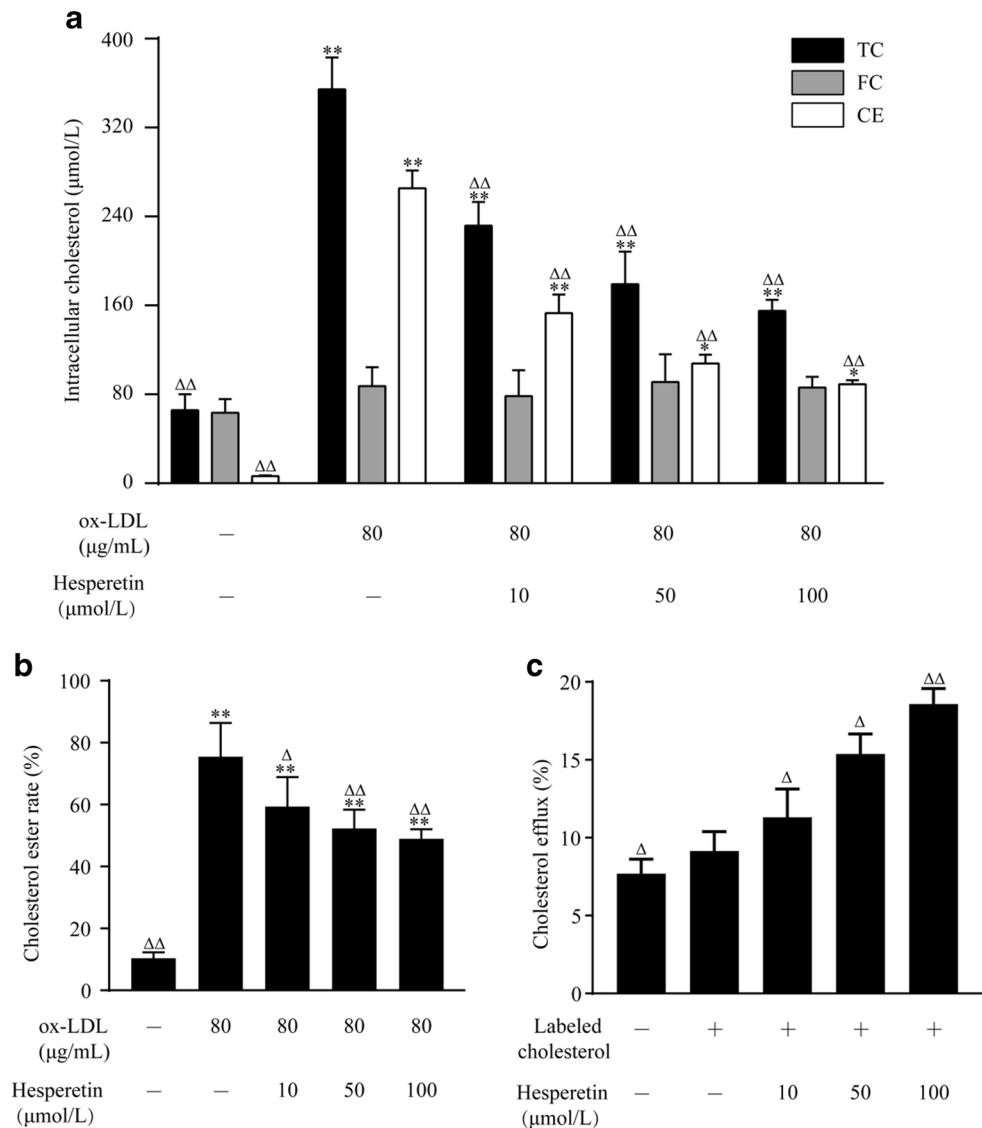


**Fig. 2** Effects of different hesperetin concentrations on the viability of THP-1 macrophages. **(a)** Cells were treated with gradient dilutions of hesperetin (0–1000  $\mu\text{mol/L}$ ) and subjected to cell viability experiments using the MTT method. The effect of hesperetin on lipid accumulation in THP-1 macrophage foam cells. THP-1 macrophages were treated with 80  $\mu\text{g/mL}$  ox-LDL alone or in combination with hesperetin (10  $\mu\text{mol/L}$ , 50  $\mu\text{mol/L}$  and 100  $\mu\text{mol/L}$ ) for 24 h. Control cells were cultured for the same time without any treatments. **(b)**

Representative microscopy images of Oil Red O-stained lipid droplets at a magnification of  $\times 200$ . **(c)** Semiquantitative analysis of intracellular lipid contents based on the absorbance of intracellular dye extracted with isopropanol at 492 nm. The results are reported as the means  $\pm$  SD from five independent experiments performed in triplicate. \* $p < 0.05$  and \*\* $p < 0.01$  compared with control;  $\Delta p < 0.05$  and  $\Delta\Delta p < 0.01$  compared with treatment with ox-LDL alone



**Fig. 3** Effects of hesperetin on cholesterol accumulation and cholesterol efflux in THP-1 macrophage foam cells. THP-1 macrophages were treated with 80  $\mu\text{g/mL}$  ox-LDL or labeled cholesterol alone or in combination with hesperetin (10  $\mu\text{mol/L}$ , 50  $\mu\text{mol/L}$  and 100  $\mu\text{mol/L}$ ) for 24 h. Control cells were cultured for the same time without any treatments. **(a)** The levels of total cholesterol (TC), free cholesterol (FC) and cholesterol ester (CE) in THP-1 macrophage foam cells were measured using an enzymatic method. **(b)** The cholesterol esterification rate (CER) is calculated as the ratio of CE to TC levels. **(c)** A commercial assay was used to determine cholesterol efflux as described above. The results are presented as the means  $\pm$  SD from five independent experiments each performed in triplicate. \* $p < 0.05$  and \*\* $p < 0.01$  compared with control;  $\Delta p < 0.05$  and  $\Delta\Delta p < 0.01$  compared with treatment with ox-LDL or labeled cholesterol alone



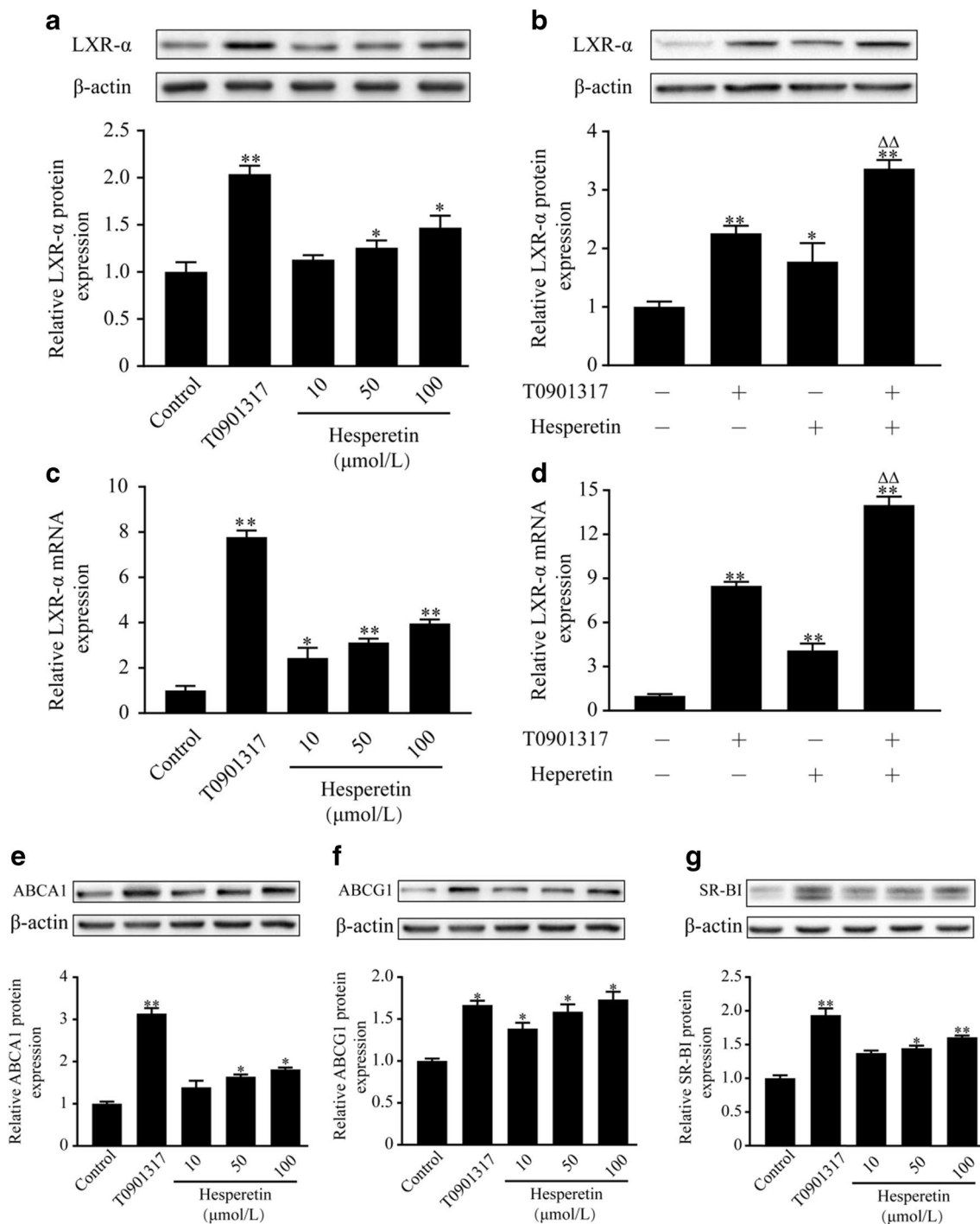
activation. The agonist (AICAR) and inhibitor (Compound C) were incubated with cells. AICAR increased the levels of the LXR $\alpha$  protein in the cells stimulated with T0901317 or hesperetin (Fig. 5c). Meanwhile, Compound C decreased the levels of the LXR $\alpha$  protein in cells stimulated with T0901317 or hesperetin (Fig. 5d). Similar results were obtained for the mRNA level (Fig. 5e and f).

### Transfection of the AMPK $\alpha$ 1/ $\alpha$ 2 siRNA decreased hesperetin-induced activation of LXR $\alpha$ signal, inhibition of foam cell formation, and promotion of cholesterol efflux

THP-1 macrophages were transfected with a specific AMPK $\alpha$ 1/ $\alpha$ 2 siRNA to further validate the role of AMPK in the hesperetin-induced upregulation of LXR $\alpha$  and its target genes. A control siRNA that will not lead to a specific decrease in mRNA expression was used as a control

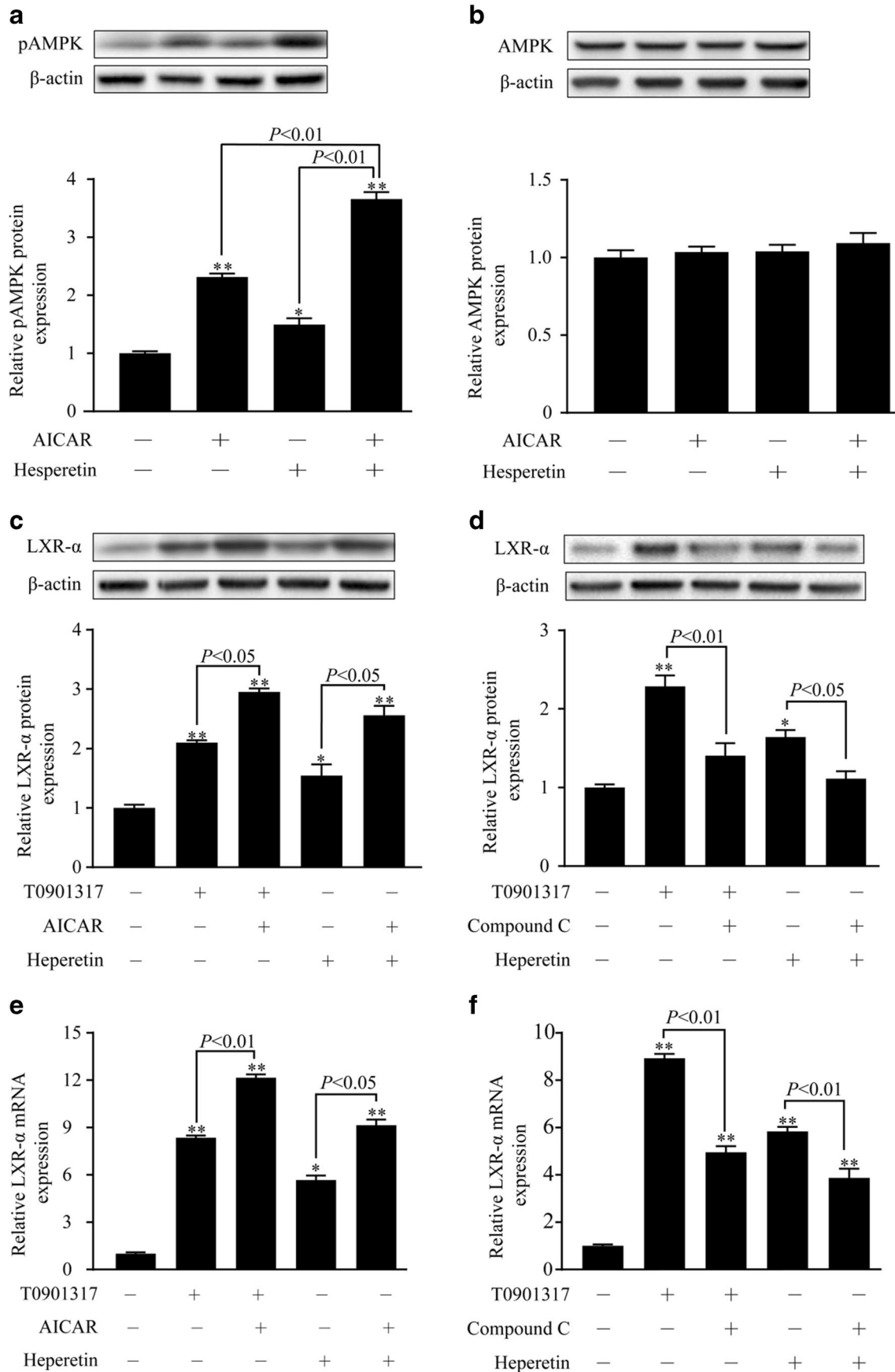
(Fig. 6a). In control siRNA group, hesperetin significantly increased the levels of the LXR $\alpha$  mRNA and its downstream targets including ABCA1, ABCG1 and SR-BI. Meanwhile, the accumulative effects were observed when cells were treated with the combination of T0901317 and hesperetin (Fig. 6c-f). In line with this result, the accumulative effects were also observed in intracellular lipid contents and cholesterol efflux when cells were treated with T0901317 combined with hesperetin (Fig. 7a-c).

After transfection with the AMPK $\alpha$ 1/ $\alpha$ 2 siRNA, the expression of the AMPK $\alpha$ 1 and AMPK $\alpha$ 2 mRNAs and the level of the pAMPK protein were significantly decreased (Fig. 6a and b). The transfection of this siRNA also reduced the expression of the LXR $\alpha$  mRNA and its target genes, including ABCA1, ABCG1 and SR-BI, compared with cells transfected with the control siRNA. Similar changes were observed in cells treated with T0901317 or hesperetin (Fig. 6c-f). In line with these results, transfection of



**Fig. 4** Effects of hesperetin on LXR $\alpha$  signal in THP-1 macrophages. THP-1 macrophages were treated with hesperetin (10  $\mu$ mol/L, 50  $\mu$ mol/L, 100  $\mu$ mol/L) and/or T0901317 (10  $\mu$ mol/L) for 24 h. Control cells were cultured for the same time without any treatments. Total proteins and RNAs were extracted and analyzed using western blot and real-time RT-PCR, respectively. **(a and c)** The effects of hesperetin on the levels of the LXR $\alpha$  protein and mRNA. **(b and d)** The

best effective concentration of hesperetin of 100  $\mu$ mol/L was used to further validate the hesperetin-induced activation of LXR $\alpha$ . **(e–g)** The effects of hesperetin on the levels of the ABCA1, ABCG1, and SR-BI proteins. The results are presented as the means  $\pm$  SD from three independent experiments performed in triplicate. \*  $p < 0.05$  and \*\*  $p < 0.01$  compared with control;  $\Delta p < 0.05$  and  $\Delta\Delta p < 0.01$  compared with treatment with T0901317





**Fig. 5** The role of AMPK in hesperetin-induced LXR $\alpha$  activation in THP-1 macrophages. THP-1 macrophages were treated with hesperetin (100  $\mu\text{mol/L}$ ) or T0901317 (10  $\mu\text{mol/L}$ ) in the presence or absence of AICARA or Compound C. Control cells were cultured for the same time without any treatments. Total proteins and RNAs were extracted and analyzed using western blot and real-time RT-PCR, respectively. (a and b) The levels of the phosphorylated AMPK and total AMPK proteins. (c and e) The effects of AMPK activation on the increase in the levels of the LXR $\alpha$  protein and mRNA induced by T0901317 or hesperetin. (d and f) The effects of AMPK inhibition on the increase in the levels of the LXR $\alpha$  protein and mRNA induced by T0901317 or hesperetin. The results are presented as the means  $\pm$  SD from three independent experiments performed in triplicate. \* $p < 0.05$  and \*\* $p < 0.01$  compared with control

the AMPK $\alpha 1/\alpha 2$  siRNA increased the lipid contents and inhibited the cholesterol efflux in THP-1 macrophage foam cells (Fig. 7a-c). Meanwhile, the effects of hesperetin and/or T0901317 on foam cell formation and cholesterol efflux were almost abolished by transfection of AMPK $\alpha 1/\alpha 2$  siRNA (Fig. 7a-c). These outcomes suggest that AMPK is implicated in hesperetin-induced activation of LXR $\alpha$  signal and LXR $\alpha$  signal-mediated inhibition of foam cell formation and cholesterol efflux.

## Discussion

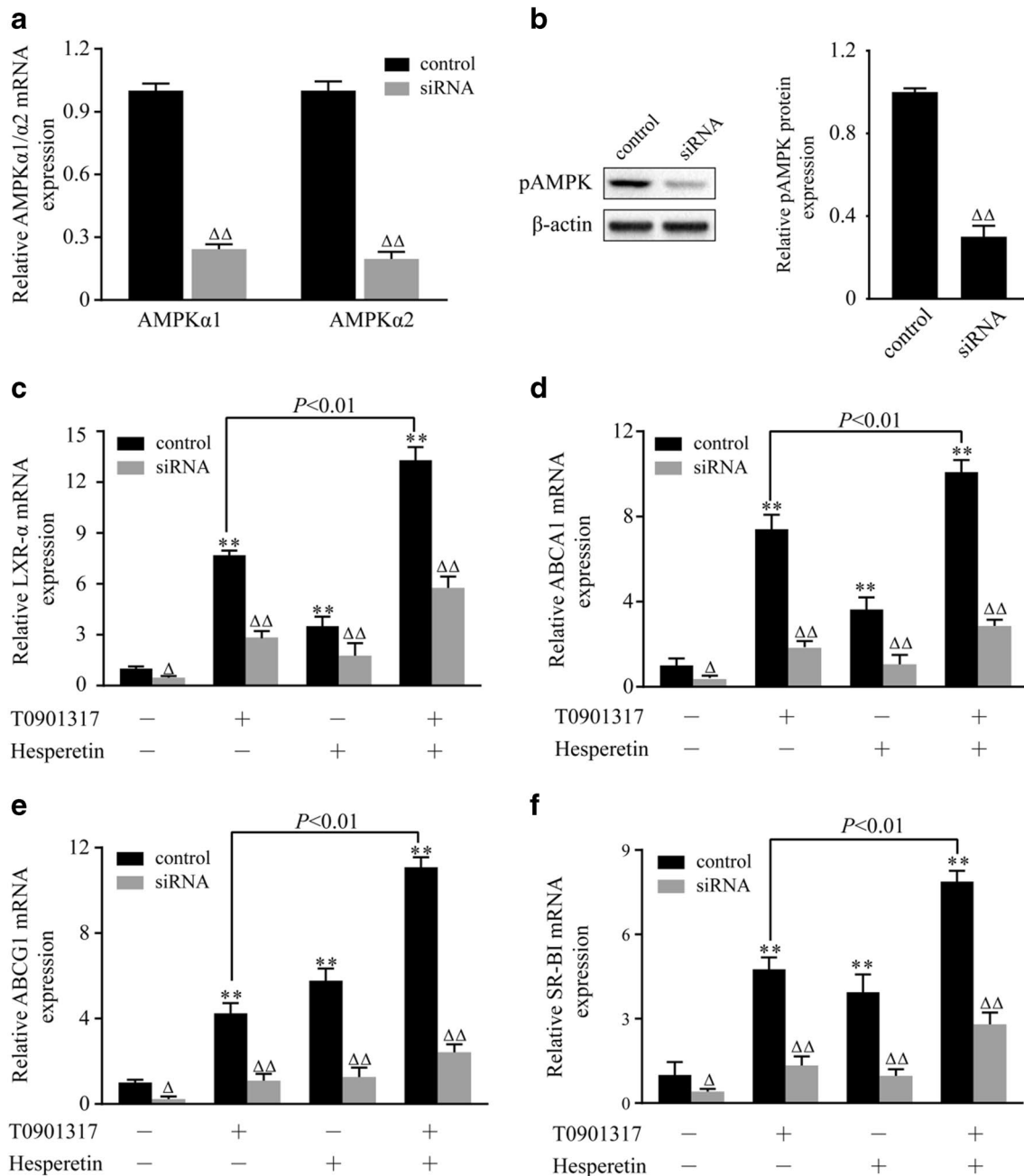
In present study, we showed for the first time that hesperetin activated AMPK in THP-1 macrophages. This activation upregulated LXR $\alpha$  and its downstream targets, including ABCA1, ABCG1 and SR-BI, to significantly inhibit foam cell formation, reduce lipid and cholesterol accumulation, and promote cholesterol efflux in THP-1 macrophages.

The early stages of atherosclerosis are characterized by lipoprotein cholesterol accumulation and foam cell formation within blood vessel walls [4]. The incidence of cardiovascular events in patients enrolled in the Dallas Heart Study was inversely correlated with cholesterol efflux [22]. According to numerous studies, hesperetin possesses hypolipidemic, anti-inflammatory and endothelial cell-protective properties [10, 21]. As shown in a recent study by Noriko Sugawara and colleagues, hesperetin evidently attenuates the development of atherosclerotic lesions in apolipoprotein E knockout mice [25]. However, the potential cytotoxicity of some flavonoids have been reported especially at high concentrations resulted from its pro-oxidant activity and mitochondrial toxicity [19]. In the present study, we first examined the effects of different concentrations (0–1000  $\mu\text{mol/L}$ ) of hesperetin on THP-1 macrophage activity to avoid its potential cytotoxicity at high concentration. Then the concentrations at 10, 50, 100  $\mu\text{mol/L}$  of hesperetin without toxic effects to THP-1 macrophages were selected for next experiments. We founded that hesperetin

increased cholesterol efflux from THP-1 macrophages, and reduced foam cell formation, cholesterol accumulation and cholesterol esterification in THP-1 macrophages, which might contribute to the observed anti-atherogenic and hypolipidemic effects of hesperetin.

LXR $\alpha$  has consistently been shown to be a promising anti-atherogenic target, due to its roles in inhibiting inflammation and promoting reverse cholesterol transport [15]. Activation of LXR $\alpha$  induces the expression of ABCA1, ABCG1 and SR-BI, all of which are membrane pumps exporting cholesterol to extracellular acceptors. Hesperetin has been reported to upregulate the enhancer activity of LXR and the expression of the ABCA1 mRNA in THP-1 macrophages [9]. The findings from our study are consistent with these results and added the findings that hesperetin significantly upregulated the expression of the LXR $\alpha$  mRNA and its target genes ABCG1 and SR-BI. These changes were validated at the protein level. Our findings provide two new targets, ABCG1 and SR-BI, for the effects of hesperetin on foam cells formation and cholesterol efflux. Meanwhile, unlike the preferential upregulation of ABCA1 to ABCG1 by an LXR $\alpha$  agonist, hesperetin upregulated the expression of these two transporters to a similar level. Thus, in addition to LXR $\alpha$  signaling, trans-activating factors specifically control the transcriptional regulation of ABCG1 in these cells. Additionally, the accumulative effect of combined T0901317 and hesperetin on LXR $\alpha$  signaling was observed. Similar effects have been reported for other flavonoids, such as the accumulative effect of combined T0901317 and curcumin on cholesterol efflux and the levels of ABCA1 mRNA and protein in THP-1 derived foam cells, and the upregulation of LXR $\alpha$  mRNA induced by resveratrol combined with T0901317 [17, 23].

Phosphorylation of AMPK has been shown to upregulate LXR $\alpha$  expression [2]. Some of flavonoids were considered the effective therapeutic approaches to modulate AMPK activity [11, 31]. According to a recent study by Hajar Shokri Afra1 et al., AMPK phosphorylation, an indicator of AMPK activation, was enhanced by hesperetin in HepG2 cells [24]. In the present study, we founded for the first time that hesperetin increased the phosphoralated level of AMPK in THP-1 macrophages. Meanwhile, an enhancement in phosphorylation of AMPK protein after the conjoint treatment of hesperetin and AICAR was detected, which is similar to the effect of hesperetin combined with T0901317 on LXR $\alpha$  signal. Analogous effects have been reported with other flavonoids [11, 31]. These results revealed a clear effect of hesperetin on AMPK activation in THP-1 macrophages. Based on the multiple biological functions of AMPK signaling, we suggested that hesperetin might be a promising therapeutic strategy targeting the activated AMPK signaling pathway in various pathological processes.

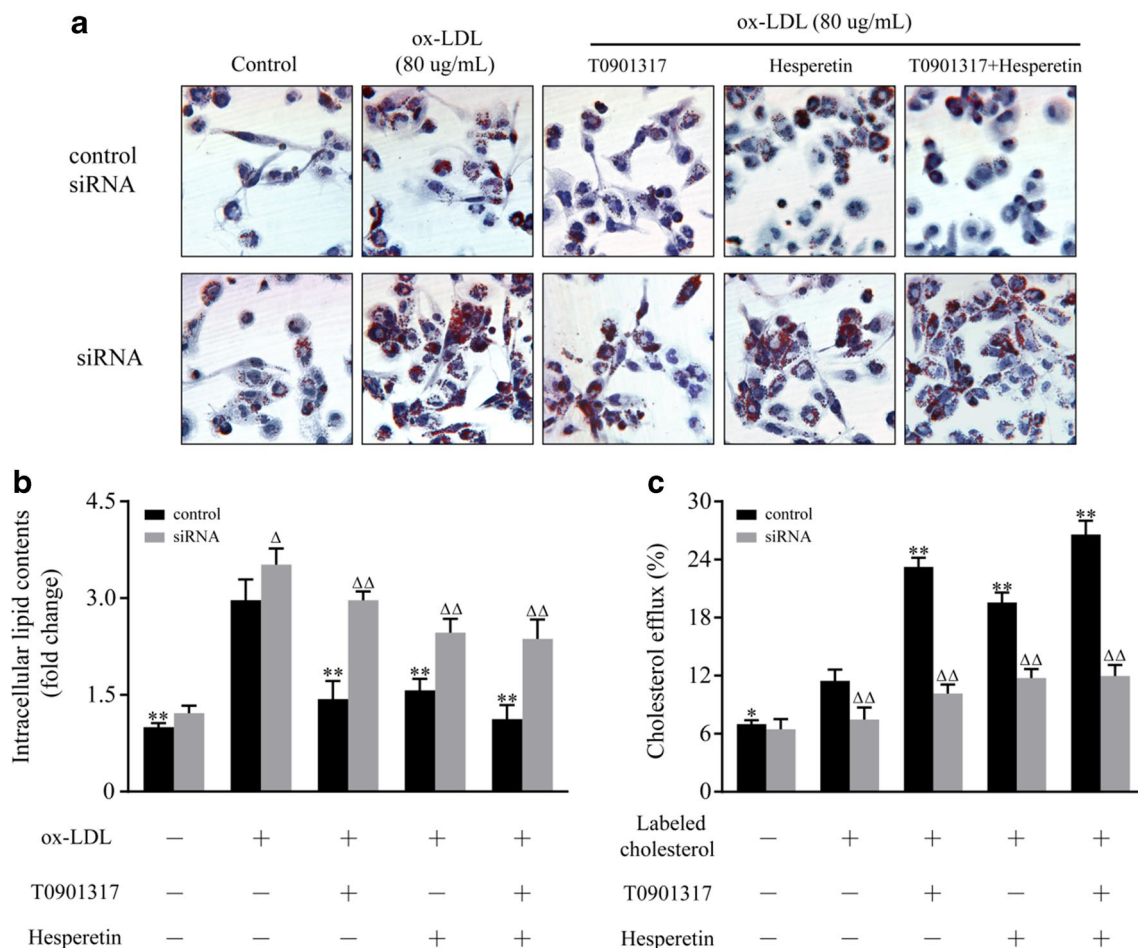


**Fig. 6** Effects of the AMPK $\alpha$ 1/ $\alpha$ 2 siRNA on hesperetin-induced increases in the expression of LXR $\alpha$  and its target genes. THP-1 macrophages were transfected with the control siRNA or siRNA targeting AMPK $\alpha$ 1/ $\alpha$ 2. Then, the cells were treated with hesperetin (100  $\mu$ mol/L) and/or T0901317 (10  $\mu$ mol/L). Control cells were cultured for the same time without any treatments. (a) The expres-

sions of the AMPK $\alpha$ 1, AMPK $\alpha$ 2 mRNAs. (b) The expression of the pAMPK protein. (c-f) The expressions of the LXR $\alpha$ , ABCA1, ABCG1, and SR-BI mRNAs. The results are reported as the means  $\pm$  SD from three independent experiments performed in triplicate. \* $p$  < 0.05 and \*\* $p$  < 0.01 compared with control;  $\Delta$   $p$  < 0.05 and  $\Delta\Delta$   $p$  < 0.01 AMPK $\alpha$ 1/ $\alpha$ 2 siRNA vs. control siRNA

We next examined the role of AMPK in hesperetin-induced LXR $\alpha$  activation. Hesperetin or T0901317 were administered in combination with or without the AMPK synthetic agonist (AICAR) or inhibitor (Compound C) to THP-1 macrophages. Then, the changes in LXR $\alpha$  expression were observed. Hesperetin-induced LXR $\alpha$  activation was

enhanced by AICAR and inhibited by Compound C. Similar results were observed in cells treated with T0901317. Furthermore, transfection with the specific AMPK $\alpha$ 1/ $\alpha$ 2 siRNA was performed to reduce phosphorylation of AMPK, which validated the role of AMPK in regulating the expression of LXR $\alpha$  and its target genes. Cells transfected with the



**Fig. 7** Effects of the AMPK $\alpha$ 1/ $\alpha$ 2 siRNA on hesperetin-induced inhibition of foam cell formation and promotion of cholesterol efflux. THP-1 macrophages were transfected with the control siRNA or siRNA targeting AMPK $\alpha$ 1/ $\alpha$ 2. Then, the cells were treated with 80  $\mu\text{g/mL}$  ox-LDL or labeled cholesterol alone or in combination with hesperetin (100  $\mu\text{mol/L}$ ) and/or T0901317 (10  $\mu\text{mol/L}$ ) for 24 h. Control cells were cultured for the same time without any treatments. (a) Representative microscopy images of Oil Red O-stained lipid

droplets at a magnification of  $\times 200$ . (b) Semiquantitative analysis of intracellular lipid contents based on the absorbance of intracellular dye extracted with isopropanol at 492 nm. (c) The cholesterol efflux was determined by a commercial assay as described above. \* $p < 0.05$  and \*\* $p < 0.01$  compared with treatment with ox-LDL or labeled cholesterol alone;  $\Delta$   $p < 0.05$  and  $\Delta\Delta$   $p < 0.01$  AMPK $\alpha$ 1/ $\alpha$ 2 siRNA vs. control siRNA

AMPK $\alpha$ 1/ $\alpha$ 2 siRNA displayed significantly reduced hesperetin and/or T0901317-induced upregulation of the LXR $\alpha$  mRNA and its targets genes, including ABCA1, ABCG1, and SR-BI. Meanwhile, the hesperetin and/or T0901317-induced inhibition of foam cell formation and promotion of cholesterol efflux were almost abolished by the siRNA transfection targeting AMPK $\alpha$ 1/ $\alpha$ 2. We therefore conclude that AMPK is implicated in hesperetin-induced activation of LXR $\alpha$  signal and LXR $\alpha$  signal-mediated inhibition of foam cell formation and promotion of cholesterol efflux. Our results were consistent with previous observations that AMPK activated LXR $\alpha$  in human macrophages [17]. However, AMPK has also been reported to inhibit LXR $\alpha$  activation to prevent lipid accumulation in HepG2 cells [18].

This discrepancy may be due to the different characteristics of pathological cell models, suggesting that AMPK may selectively regulate LXR $\alpha$  activation in different pathological process.

In conclusion, hesperetin prevents foam cell formation, reduces total cholesterol levels and the cholesterol esterification rate, and increases cholesterol efflux in THP-1 macrophages through the activation of LXR $\alpha$  signaling pathway in an AMPK-dependent manner. This new mechanism contributes to the knowledge of the beneficial effects of hesperetin on regulating cholesterol homeostasis. We postulate that similar to other flavonoids, hesperetin will naturally find its place in the routine treatment of hyperlipemia and atherosclerosis.

**Abbreviations** ABCA1, ATP-binding cassette subfamily A member 1; ABCG1, ATP-binding cassette subfamily G member 1; ABCG5, ATP-binding cassette subfamily G member 5; AMPK, Adenosine monophosphate-activated protein kinases; CE, Cholesterol ester; CER, Cholesterol esterification rate; FC, Free cholesterol; LXR $\alpha$ , Liver X receptor alpha; ox-LDL, Oxidized low-density lipoprotein; pAMPK, Phosphorylated AMPK; RCT, Reverse cholesterol transport; siRNA, Small interfering RNA; SR-BI, Scavenger receptor class B type I; TC, Total cholesterol.

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**Author contributions** The conception and design were proposed by Xuanjing Chen. Cells experiment and Molecular biology experiment was finished by Xuanjing Chen, Xiaoling Chen and Dezhi Zou. Data analysis were conducted by Xuanjing Chen. Paper was drafted by Xuanjing Chen and reviewed by Danping Xu and Huanlin Wu. Funding was secured by Huanlin Wu and Danping Xu. The final version has been approved and agreed for publication by all authors and by the Scientific Research Office of Guangdong Provincial Hospital of Chinese Medicine.

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

## Compliance with ethical standards

**Conflicts of interest** The authors report no conflicts of interest.

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