Clinical and Experimental Immunology, 2022, **209**, 316–325
https://doi.org/10.1093/cei/uxac062
Advance access publication 24 June 2022
Research Article

Research Article

Baicalein inhibits macrophage lipid accumulation and inflammatory response by activating the PPARγ/LXRα pathway

Zi-Zhen Zhang[1], Xiao-Hua Yu[2] and Wei-Hua Tan[3,*]

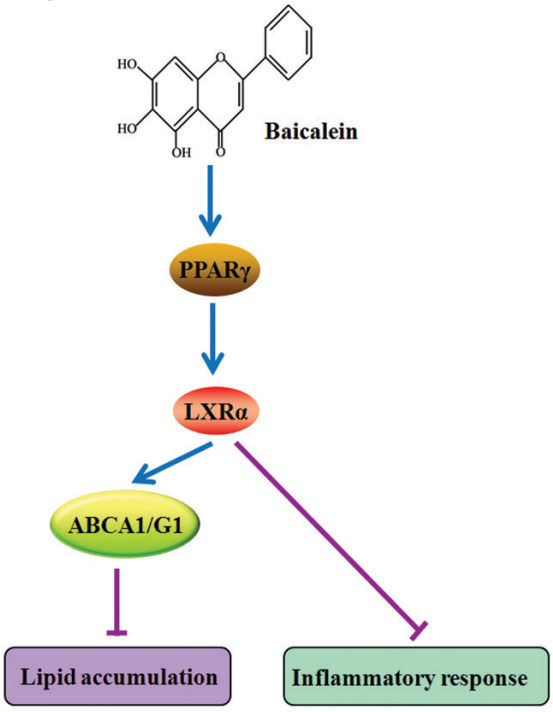
[1]School of Medicine, Hunan Polytechnic of Environment and Biology, Hengyang Hunan, China
[2]Institute of Clinical Medicine, The Second Affiliated Hospital of Hainan Medical University, Haikou, Hainan, China
[3]Emergency Department, The First Affiliated Hospital of University of South China, Hengyang, Hunan, China

[*]Correspondence: Wei-Hua Tan, Emergency Department, The First Affiliated Hospital of University of South China, Hengyang 421001 Hunan, China.
Email: tanweihua830928@163.com

Abstract

Lipid accumulation and inflammatory response are two major risk factors for atherosclerosis. Baicalein, a phenolic flavonoid widely used in East Asian countries, possesses a potential atheroprotective activity. However, the underlying mechanisms remain elusive. This study was performed to explore the impact of baicalein on lipid accumulation and inflammatory response in THP-1 macrophage-derived foam cells. Our results showed that baicalein up-regulated the expression of ATP binding cassette transporter A1 (ABCA1), ABCG1, liver X receptor α (LXRα), and peroxisome proliferator-activated receptor γ (PPARγ), promoted cholesterol efflux, and inhibited lipid accumulation. Administration of baicalein also reduced the expression and secretion of TNF-α, IL-1β, and IL-6. Knockdown of LXRα or PPARγ with siRNAs abrogated the effects of baicalein on ABCA1 and ABCG1 expression, cholesterol efflux, lipid accumulation as well as pro-inflammatory cytokine release. In summary, these findings suggest that baicalein exerts a beneficial effect on macrophage lipid accumulation and inflammatory response by activating the PPARγ/LXRα signaling pathway.

Graphical Abstract



Received 28 November 2021; Revised 27 May 2022; Accepted for publication 23 June 2022

Keywords: baicalein, PPARγ, LXRα, lipid accumulation, inflammatory response

Abbreviations: ABCA1: ATP binding cassette transporter A1; ABCG1: ATP binding cassette transporter G1; apoE: apolipoprotein E; TNF-α: tumor necrosis factor-α; IL: interleukin; ox-LDL: oxidized low-density lipoprotein; VSMCs: vascular smooth muscle cells; PPARγ: peroxisome proliferator-activated receptor γ; LXRα: liver X receptor α; DMSO: dimethyl sulfoxide; Dil-ox-LDL: dil dye-labeled ox-LDL; SR-A: scavenger receptor class A; HRP: horseradish peroxidase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HPLC: high-performance liquid chromatography; PBS: phosphate buffered saline; FC: free cholesterol; CE: cholesterol esterase; TC: total cholesterol; siRNAs: small interfering RNAs; ELISA: enzyme-linked immunosorbent assay; qRT-PCR: quantitative real-time PCR.

Introduction

Atherosclerosis is a lipid-driven inflammatory disease and constitutes the pathological basis of coronary artery disease and cerebrovascular disease. As the most abundant immune cell type in atherosclerotic lesions, macrophages play a critical role throughout all stages of this disease, from lesion formation to plaque rupture. After accumulating large amounts of lipids, macrophages become foam cells, a hallmark of early-stage atherosclerotic lesions [1]. ATP binding cassette transporter A1 (ABCA1) and G1 (ABCG1) belong to transmembrane proteins and their major function is to mediate intracellular cholesterol efflux. Previous studies from our group and others have demonstrated that promoting ABCA1- and ABCG1-dependent cholesterol release inhibits lipid accumulation in THP-1 macrophages and reduces atherosclerotic lesion area in apolipoprotein E-deficient (apoE$^{-/-}$) mice [2–4]. Interestingly, macrophage foam cells can also generate multiple pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6. The release of these inflammatory mediators not only further exacerbates lipid accumulation but also contributes to the expansion of the necrotic core [5, 6]. Despite the use of statins has considerably improved the clinical outcomes of hyperlipidemia patients, the residual risk is still higher due to the unsolved inflammation [7, 8]. Thus, seeking novel drugs with anti-inflammatory and lipid-lowering effects is of critical importance to effectively prevent and treat atherosclerotic cardiovascular disease.

Baicalein (5,6,7-trihydroxyflavone, Fig. 1A) is a major flavonoid extracted from the root of *Scutellaria baicalensis* Georgi, a medicinal plant traditionally used in oriental medicine. This compound has a variety of pharmacological activities, including anti-inflammation, anti-oxidative stress, and anti-infection [9–11]. Administration of baicalein has been shown to protect against acute ischemia/reperfusion injury, hypertension, heart failure, and myocardial infarction in animal models [12–15]. Recently, Chan et al. reported that baicalein ameliorates oxidized low-density lipoprotein (ox-LDL)-induced endothelial dysfunction by inhibiting the inflammatory response and cell apoptosis [16]. In addition, baicalein treatment markedly suppresses the proliferation of vascular smooth muscle cells (VSMCs), a critical event during atherogenesis [17]. These findings suggest that baicalein may exert an atheroprotective role. However, the molecular mechanisms for its anti-atherogenic action remain largely unknown.

Peroxisome proliferator-activated receptor γ (PPARγ) and liver X receptor α (LXRα) belong to the members of the nuclear receptor superfamily. LXRα interacts with retinoid X receptor to form an obligate heterodimer that can recognize LXR response elements in the ABCA1 and ABCG1 promoter region to stimulate gene transcription [18]. There is increasing evidence that activation of the PPARγ/LXRα signaling pathway mitigates lipid accumulation in THP-1 macrophages and protects against atherosclerosis in apoE$^{-/-}$ mice by up-regulating ABCA1 and ABCG1 expression [19–21]. Of note, activation of this pathway can also inhibit the production and secretion of pro-inflammatory cytokines [22, 23]. Several lines of evidence have demonstrated that baicalein serves as an activator of PPARγ in human colon cancer cells [24] and hepatic stellate cells [25]. It is still unclear, however, whether baicalein can activate the PPARγ/LXRα pathway to increase ABCA1 and ABCG1 expression and antagonize inflammatory response in macrophages.

The present study aimed to observe the influence of baicalein on macrophage lipid accumulation and inflammatory response and to explore the underlying mechanisms. We found that baicalein enhanced ABCA1- and ABCG1-dependent cholesterol efflux and reduced pro-inflammatory cytokine secretion from THP-1 macrophage-derived foam cells by activating the PPARγ/LXRα pathway.

Materials and methods

Reagents and antibodies

Baicalein (HPLC>98%) was purchased from Sigma–Aldrich (St. Louis, MO, USA), solved in dimethyl sulfoxide (DMSO), and then stored at −20°C in the dark. Ox-LDL and dil dye-labeled ox-LDL (Dil-ox-LDL) were provided by Yiyuan Biotechnologies (Guangzhou, China). Rabbit antibodies against ABCA1, ABCG1, CD36, LXRα, and PPARγ, β-actin, and histone H3 are the products of Abcam (Cambridge, UK). Mouse antibody against scavenger receptor class A (SR-A; R&D systems), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit or mouse IgG (Proteintech Group, Chicago, IL, USA) were obtained as indicated.

Cell culture

THP-1 monocytes (Cell Bank of Chinese Academy of Science, Shanghai, China) were cultured in RPMI 1640 medium (Sigma–Aldrich) containing 10% fetal bovine serum (Sigma–Aldrich) and 2% penicillin–streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. After 24 h of incubation with 160 nM phorbol-12-myristate acetate (Sigma–Aldrich), THP-1 monocytes were differentiated into macrophages. Then, THP-1 macrophages were cultured in the fresh 10% FBS/RPMI 1640 medium for 12 h. To induce foam cell formation, THP-1 macrophages were treated with 50 μg/ml ox-LDL for 48 h in a serum-free medium.

Detection of cell viability

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as previously described [26]. Briefly, THP-1 macrophage-derived foam cells were seeded in 96-well plates at a density of 2×10^6 cells/well. When the cells were 80% confluent, the cells were treated with various concentrations of baicalein (0, 25, 50, 100, or 200 μM) for 24 h or 100 μM baicalein for

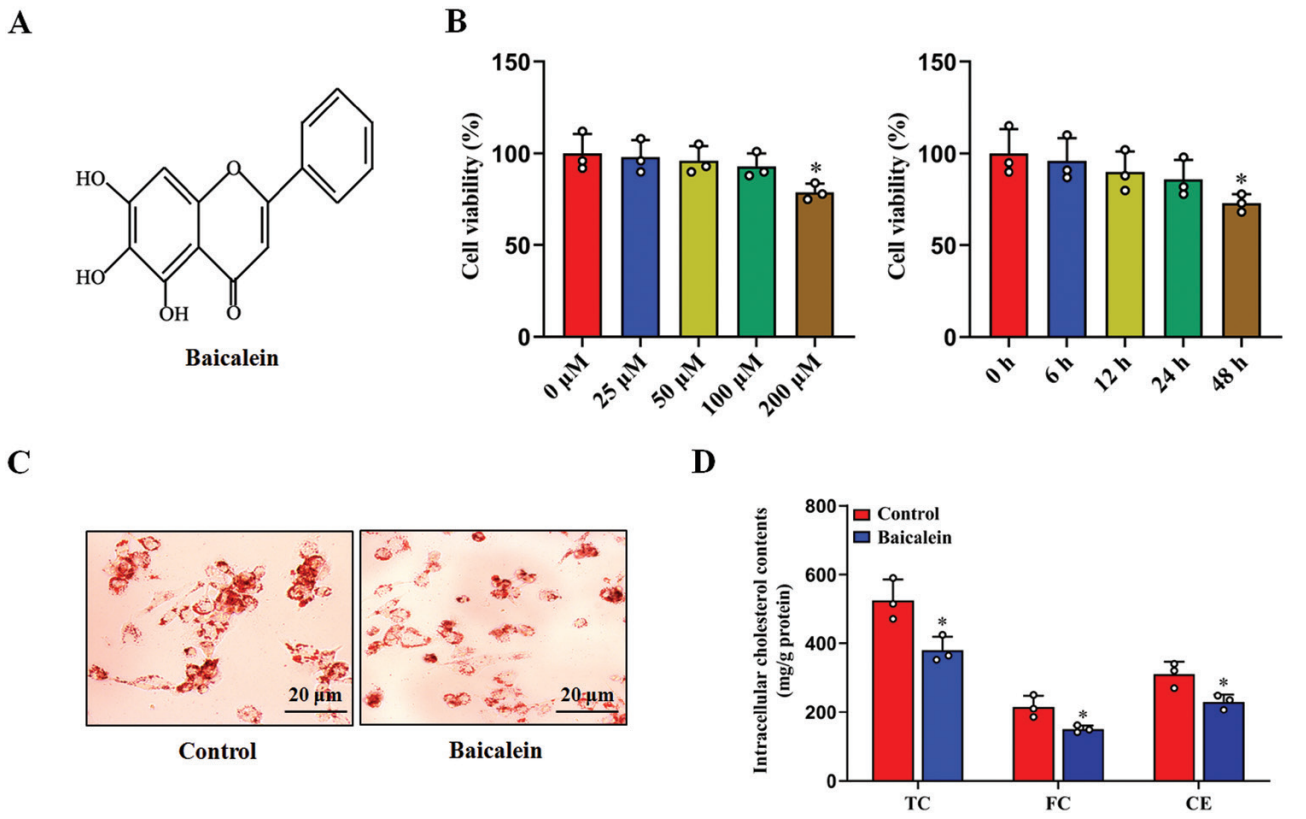


Figure 1: effects of baicalein on cell viability and intracellular lipid accumulation. (A) Chemical structure of baicalein. (B) THP-1 macrophage-derived foam cells were treated with the indicated concentrations of baicalein for 24 h or with 100 μM baicalein for various times. MTT assay was employed to evaluate cell viability. (C–D) THP-1 macrophage-derived foam cells were incubated with PBS or 100 μM baicalein for 24 h. (C) The cells were fixed and then stained with Oil Red O to assay intracellular lipid deposition. The magnification of each panel is 200×. (D) Intracellular TC, FC, and CE amounts were detected by HPLC. Data are expressed as mean ± SD from three independent experiments. *$P < 0.05$ vs 0 μM or 0 h groups.

the indicated time (0, 6, 12, 24, or 48 h). Then, cells were incubated with 10 μl of 5 mg/ml MTT (Sigma–Aldrich) at 37°C for 4 h. The medium was carefully removed and 100 μL DMSO was added to each well to dissolve the formazan crystals. The absorbance was detected at 570 nm using a synergy H1 hybrid microplate reader (Winooski, VT, USA).

Oil Red O staining

THP-1 macrophage-derived foam cells were treated with phosphate-buffered saline (PBS) or 100 μM baicalein for 24 h. After washing with PBS, cells were fixed with 4% paraformaldehyde for 10 min. Subsequently, cells were stained with Oil Red O solution (Solarbio, Beijing, China) in the dark for 20 min, washed with 60% isopropanol for 10 s, and photographed using an inverted fluorescent microscope (Olympus, Tokyo, Japan).

High-performance liquid chromatography (HPLC) analysis

After the indicated treatments, THP-1 macrophage-derived foam cells were broken in 1 ml of 0.9% NaCl by an ultrasonic processor after washing. BCA Protein Assay Kit (Beyotime, Shanghai, China) was employed to detect the protein concentration of cell lysates. Cholesterol was extracted by n-hexane-isopropanol (3:2, V/V), dissolved in isopropanol (50 mg/ml), and stored at –20 °C. Meanwhile, cholesterol standard calibration solution ranging from 0 to 50 mg/ml was prepared. The reaction mixture containing $MgCl_2$ (500 mM), Tris–HCl (500 mM, pH = 7.4), dithiothreitol (10 mM) and 5% NaCl, was added to 100 μl of cholesterol standard calibration solution and cell solution. Then, 0.4 U cholesterol oxidase was supplemented to detect free cholesterol (FC) content, and 0.4 U cholesterol oxidase combined with 0.4 U cholesterol esterase (CE) was supplemented for measurement of total cholesterol (TC) amount. After incubating at 37°C for 30 min, the reaction was terminated, and the supernatant was collected to measure the absorbance at 216 nm. CE was estimated by subtracting FC from TC. Data were analyzed using Total Chrom software (Perkin Elmer, Waltham, MA, USA).

Transfection of small interfering RNAs (siRNAs)

LXRα siRNA (forward, 5′-GGCUGCAAGUGGAAUU CAUTT-3′; reverse, 5′-AUGAAUUC CACUUGCAGCCTT-3′), PPARγ siRNA (forward, 5′-GGAUGCAAGGGUUUCUUCC TT-3′; reverse, 5′-GGAAGAAACCCUUGCAUCCTT-3′), and scrambled siRNA as negative control (forward, 5′-UU CUCCGAACGUGUCACGUTT-3′; reverse, 5′-ACGUGACAC GUUCGGAGAA TT-3’) were designed and synthesized by GenePharma (Shanghai, China). The siRNAs (40 nM) were transfected into THP-1 macrophage-derived foam cells (2×10^6 cells/well) using lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. After 48 h, transfection efficiency was measured by western blot.

Detection of intracellular cholesterol efflux

Cholesterol efflux was determined as described elsewhere [27]. In brief, THP-1 macrophage-derived foam cells were incubated with PBS or 100 μM baicalein for 24 h and then labelled with 5 μCi/ml [^{3}H]-cholesterol for an additional 24 h. Cells were washed three times with PBS, followed by incubation with serum-free RPMI 1640 medium for 2 h at 37°C. Serum-free medium was removed, and cells were cultured in 2 ml of RPMI 1640 medium containing 20 μg/ml apoA-I (Sigma-Aldrich) or 50 μg/ml HDL (Sigma–Aldrich) overnight. The radioactivity of [^{3}H]-cholesterol in the medium and cells was detected using a liquid scintillation counter, respectively. The percent cholesterol efflux was calculated as the ratio of radioactivity in the medium to total radioactivity (medium + cells).

Dil-ox-LDL uptake assay

For visualization of ox-LDL uptake, THP-1 macrophage-derived foam cells were treated with PBS or 100 μM baicalein for 24 h and then incubated with 10 μg/ml Dil-ox-LDL for 4 h at 37°C. After washing three times with PBS, cells were viewed and imaged by an inverted fluorescence microscopy. Fluorescence intensity was detected using Image Pro Plus software (Diagnostic Instruments, USA).

Enzyme-linked immunosorbent assay (ELISA)

At the end of treatment, a cell culture supernatant was collected to detect IL-1β, IL-6, and TNF-α amounts using the commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance at 450 nm was determined by the iMark™ microplate reader (Bio-Rad, Hercules, CA, USA).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from THP-1 macrophage-derived foam cells using TRIzol reagent (Invitrogen). RNA (500 ng) from each sample was reverse-transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Takara, Kyoto, Japan). Then, qRT-PCR was performed using SYBR Green Real-Time PCR Master Mix (Promega) and the Mx3005P system (Agilent Technologies, US). The samples were incubated at 95°C for 10 min, followed by 40 cycles under the following conditions: 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The primers for qRT-PCR were synthesized by Sangon Biotech (Shanghai, China), and their sequences are presented in Table 1. The $2^{-\Delta\Delta}$Ct method was used to analyze the relative expression levels of target genes, with GAPDH as an internal control.

Table 1: primer sequences for qRT-PCR

Genes	Sequences (5′-3′)
ABCA1	ACCCACCCTAT GAACAACATGA (forward)
	GAGTCGGGTAACGGAAACAGG (reverse)
ABCG1	ATTCAGGG ACCTTTCCTATTCGG (forward)
	CTCACCACTATTGAACTTCCCG (reverse)
CD36	AAGCCAG GTATTGCAGTTCTTT (forward)
	GCATTTGCTGATGT CTAGCAC A (reverse)
SR-A	GCAGTG GGATCACTTTCACAA (forward)
	AGCTGTCATTGAGCGAGCA TC (reverse)
LXRα	CCTTCAG AACCCACAGAGATCC (forward)
	ACGCTGCATAGCTCGTT CC (reverse)
PPARγ	TGGAATTAGATG ACAGCGACTTGG (forward)
	CTGGAGCAGCTTGGCAAACA (reverse)
TNF-α	CTCTTCTGCCTGCTGCACTTTG (forward)
	ATGGGCTACAGGCTTGTCACTC (reverse)
IL-1β	CCACAGACCTTCCAGGAGAATG (forward)
	GTGCAGTTCAGTGATCGTACAGG (reverse)
IL-6	AGACAGCCACTCACCTCTTCAG (forward)
	TTCTGCCAGTGCCTCTTTGCTG (reverse)
GAPDH	TGTGGGCATC AATGGATTTGG (forward)
	ACACCATGTATTCCGG GTCAAT (reverse)

Protein extraction and western blot

After the indicated treatments, cells were harvested and then lysed by the RIPA buffer (Beyotime) containing 0.1 mmol/l PMSF on ice for 30 min to extract total proteins. The cytoplasmic and nuclear proteins were extracted by the corresponding protein extraction kits (Beyotime). After centrifugation, the supernatant was collected and protein concentrations were determined using BCA Protein Assay Kit (Beyotime). Protein samples (20 μg/well) were subjected to SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk for 2 h, PVDF membranes were incubated with antibodies against ABCA1 (ab7360, 1:200), ABCG1 (ab52617, 1:1000), CD36 (ab133625, 1:500), SR-A (AF1797, 1:1000), LXRα (ab176323, 1:2000), PPARγ (ab178860, 1:500), β-actin (ab115777, 1:3000), or histone H3 (ab176842, 1:3000) overnight at 4°C. The membranes were washed and further incubated with HRP-conjugated goat anti-rabbit IgG (SA00001-2, 1:5000) or mouse IgG (SA00001-1, 1:5000) at room temperature for 2 h. The protein bands were visualized with the BeyoECL Plus kit (Beyotime) and quantified using Gel-Pro software (version 4.0). β-actin was used as an internal control.

Statistical analysis

All data are expressed as the mean ± SD from three independent experiments and analyzed by GraphPad Prism 8.0 software. An unpaired two-tailed Student's *t*-test was used to compare the differences between the two groups. Multiple comparisons were made among ≥ three groups using one-way ANOVA followed by the Bonferroni post hoc test. A *P* value less than 0.05 was considered statistically significant.

Results

Baicalein inhibits lipid accumulation in THP-1 macrophage-derived foam cells

To determine the effects of baicalein on cell viability, THP-1 macrophage-derived foam cells were exposed to various concentrations of baicalein (0, 25, 50, 100, or 200 μM) for 24 h or 100 μM baicalein for the indicated time (0, 6, 12, 24, or 48 h). The MTT results showed that there was no cytotoxicity when cells were treated with 25–100 μM baicalein for 6–24 h (Fig. 1B). We thus selected 100 μM baicalein to treat

cells for 24 h in the subsequent experiments. By using Oil Red O staining, we found that lipid droplets were smaller and fewer in the baicalein group compared with the control group (Fig. 1C). Consistently, administration of baicalein markedly reduced intracellular TC, FC, and CE amounts (Fig. 1D). These data suggest that baicalein exerts an inhibitory effect on macrophage lipid accumulation.

Baicalein up-regulates ABCA1 and ABCG1 expression and promotes cholesterol efflux in THP-1 macrophage-derived foam cells

Decreased cholesterol efflux mediated by ABCA1 and ABCG1 is a major cause leading to macrophage lipid accumulation. To reveal the underlying mechanism by which baicalein protects against lipid accumulation, we examined the effects of baicalein on these two transporter expressions and cholesterol efflux. The qRT-PCR and western blot results demonstrated that treatment with baicalein dramatically enhanced the mRNA and protein levels of ABCA1 and ABCG1 in THP-1 macrophage-derived foam cells (Fig. 2A). Moreover, baicalein up-regulated the protein expression of ABCA1 and ABCG1 in a time- and concentration-dependent manner (Supplementary Fig. 1A and B). Accordingly, the efficiency of cholesterol efflux to apoA-I and HDL was significantly increased in response to baicalein (Fig. 2B). CD36- and SR-A-mediated cholesterol influx also contributes to intracellular lipid deposition [28]. However, administration of baicalein had no influence on CD36 and SR-A expression (Fig. 2C) as well as Dil-ox-LDL uptake (Fig. 2D). To summarize, promoting ABCA1- and ABCG1-dependent cholesterol efflux is necessary for baicalein-induced prevention of macrophage lipid accumulation.

LXRα is involved in baicalein-induced enhancement of ABCA1 and ABCG1 expression

LXRα is known to play a key role in stimulating ABCA1 and ABCG1 transcription. To determine whether baicalein-induced up-regulation of ABCA1 and ABCG1 expression is mediated by LXRα, we first measured the expression of LXRα in THP-1 macrophage-derived foam cells treated with baicalein using qRT-PCR and western blot. As expected, baicalein dramatically augmented the mRNA and protein levels of LXRα (Fig. 3A). Further, baicalein time- and concentration-dependently increased LXRα protein expression (Supplementary Fig. 1A and B). We then used siRNA to silence the endogenous expression of LXRα in THP-1 macrophage-derived foam cells. In comparison with the control group, the LXRα protein levels were inhibited by 72% in the LXRα siRNA group (Fig. 3B). In the presence of LXRα knockdown, baicalein partially restored ABCA1 and ABCG1 expression (Fig. 3C and D) as well as cholesterol efflux (Fig. 3E). The decrease in intracellular TC, FC, and CE levels by baicalein was suppressed by pretreatment with LXRα siRNA (Supplementary Fig. 2A). These findings suggest that baicalein increases ABCA1 and ABCG1 expressions, promotes cholesterol efflux, and inhibits macrophage lipid accumulation in an LXRα-dependent manner.

PPARγ is required for induction of LXRα, ABCA1, and ABCG1 expression by baicalein

PPARγ is an upstream effector of LXRα. Our results showed that baicalein treatment dramatically up-regulated the mRNA and protein expression of PPARγ in THP-1 macrophage-derived foam cells (Fig. 4A). Moreover, baicalein up-regulated PPARγ protein expression in a time- and concentration-dependent manner (Supplementary Fig. 1A and B). The nuclear translocation of PPARγ was also promoted by baicalein (Fig. 4B). We then transfected THP-1 macrophage-derived foam cells with PPARγ siRNA or scrambled siRNA and found that PPARγ siRNA treatment reduced PPARγ protein levels by 68% (Fig. 4C). This indicates that PPARγ siRNA can effectively silence endogenous PPARγ expression. Importantly, the expression LXRα, ABCA1, and ABCG1 and intracellular cholesterol export were partially restored when

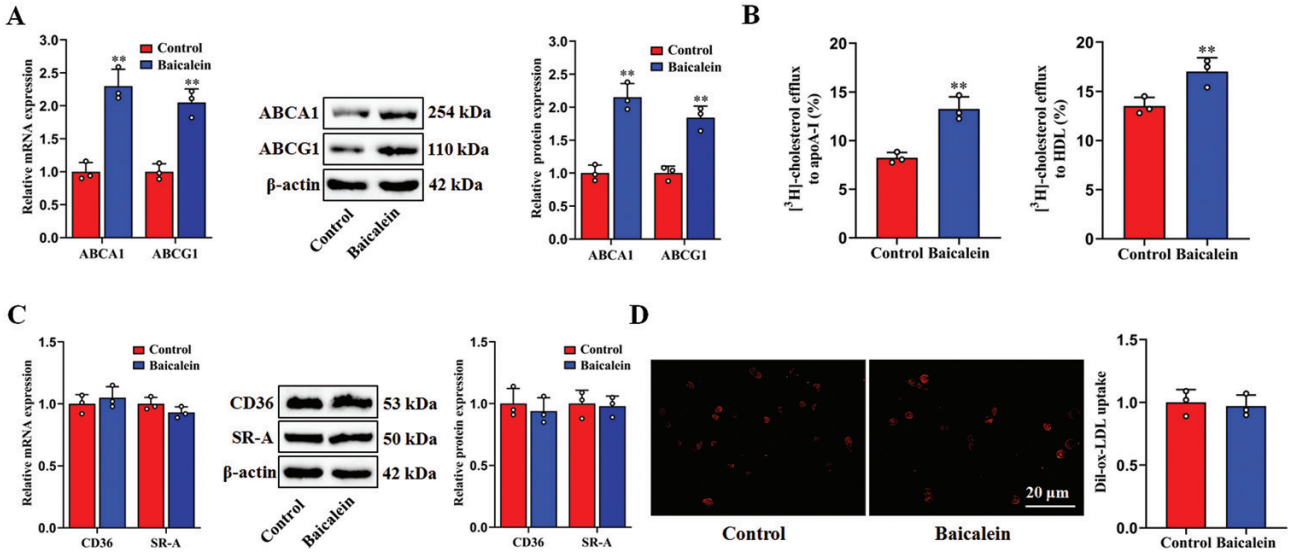


Figure 2: Baicalein facilitates ABCA1- and ABCG1-dependent cholesterol efflux. (A–D) THP-1 macrophage-derived foam cells were exposed to PBS or 100 μM baicalein for 24 h. (A) The mRNA and protein levels of ABCA1 and ABCG1 were determined by qRT-PCR and western blot, respectively. (B) Cholesterol efflux to apoA-I and HDL was analyzed by the liquid scintillation counting method. (C) Detection of CD36 and SR-A expression using qRT-PCR and western blot. (D) Representative fluorescent images of Dil-ox-LDL uptake. The magnification of each panel is 200×. All data are presented as the mean ± SD from three independent experiments. $^{**}P < 0.01$ vs. control group.

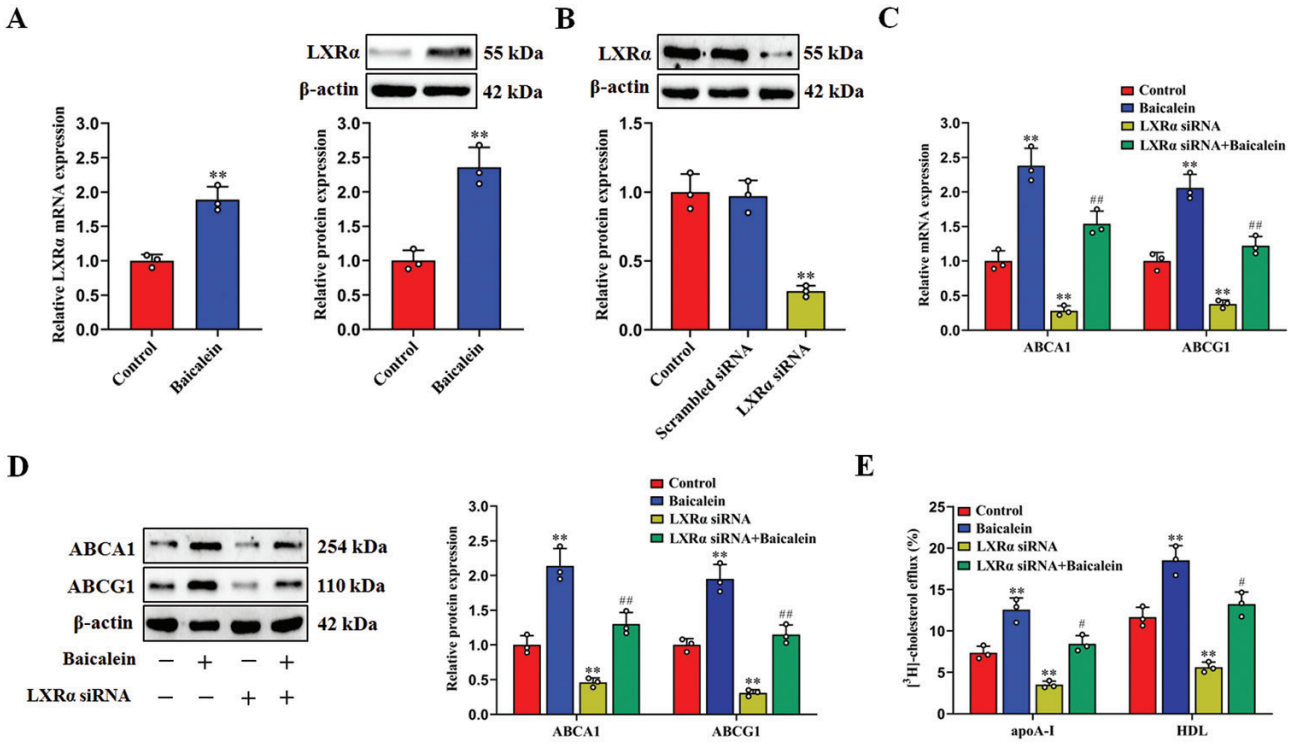


Figure 3: involvement of LXRα in the induction of ABCA1 and ABCG1 expression by baicalein. (A) THP-1 macrophage-derived foam cells were incubated with PBS or 100 μM baicalein for 24 h, followed by measurement of LXRα expression using qRT-PCR and western blot. (B–E) THP-1 macrophage-derived foam cells were transfected with scrambled siRNA or LXRα siRNA for 48 h and then incubated with or without 100 μM baicalein for 24 h. (B) Cell lysates were subjected to western blot analysis to assess the protein levels of LXRα. (C–D) The expression of ABCA1 and ABCG1 were evaluated using qRT-PCR and western blot. (E) Cholesterol efflux to apoA-I and HDL was detected by the liquid scintillation counting method. Data are expressed as mean ± SD from three independent experiments. $^{**}P < 0.01$ vs control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs baicalein group.

THP-1 macrophage-derived foam cells were treated with PPARγ siRNA and baicalein (Fig. 4D and E). Pretreatment with PPARγ siRNA also reduced the impact of baicalein on intracellular TC, FC, and CE contents (Supplementary Fig. 2B). Interestingly, the double knockdown of PPARγ and LXRα fully reversed baicalein-induced enhancement of cholesterol efflux from THP-1 macrophage-derived foam cells (Supplementary Fig. 3). These results suggest that baicalein increases LXRα, ABCA1, and ABCG1 levels, facilitates cholesterol efflux and inhibits macrophage lipid accumulation by up-regulating PPARγ expression.

Baicalein suppresses inflammatory response by activating the PPARγ/LXRα pathway in THP-1 macrophage-derived foam cells

Ox-LDL is also a stimulus of inflammation [29]. Activation of the PPARγ/LXRα pathway not only inhibits intracellular lipid accumulation but also alleviates inflammatory response. Given baicalein as an activator of this signaling cascade, we next tested its influence on pro-inflammatory cytokine production and secretion in THP-1 macrophage-derived foam cells. As shown in Fig. 5A, the mRNA expression of TNF-α, IL-1β, and IL-6 was significantly up-regulated in ox-LDL-challenged THP-1 macrophages compared with unstimulated cells, whereas this up-regulation was inhibited by baicalein treatment. Incubation with ox-LDL also dramatically increased the levels of TNF-α, IL-1β, and IL-6 in the cell culture supernatant, which was alleviated by baicalein (Fig. 5B). Importantly, pretreatment with siRNAs against LXRα or PPARγ significantly reversed the inhibitory effect of baicalein on the expression and secretion of these cytokines (Fig. 5C–F). These observations indicate that baicalein activates the PPARγ/LXRα pathway to antagonize inflammation.

Discussion

Atherosclerosis predominantly occurs in large- and medium-arteries. Lipid deposition in the subendothelial space is a major pathogenesis of atherosclerosis. Baicalein is a natural plant flavone originally isolated from the root of *Scutellaria baicalensis* Georgi, a kind of Chinese herbal medicine. Baicalein has been reported to improve endothelial dysfunction and antagonize VSMC proliferation [16, 17]. Nevertheless, very little is known about its impact on macrophage lipid accumulation. A recent study showed that treatment with baicalein inhibits intracellular lipid accumulation in 3T3-L1 preadipocytes [30]. Baicalein also diminishes TC levels in HepG2 cells [31]. Baicalin, also known as baicalein-7-glucuronide, was shown to restrain lipid deposition in macrophages [32]. Similarly, we found that administration of baicalein decreased intracellular cholesterol contents and the number of lipid droplets in THP-1 macrophage-derived foam cells. Thus, the prevention of macrophage lipid accumulation may be another important mechanism by which baicalein mitigates atherosclerosis.

It is generally known that lipid accumulation is caused by increased cholesterol efflux and/or decreased cholesterol uptake. ABCA1 mediates the initial transport of intracellular

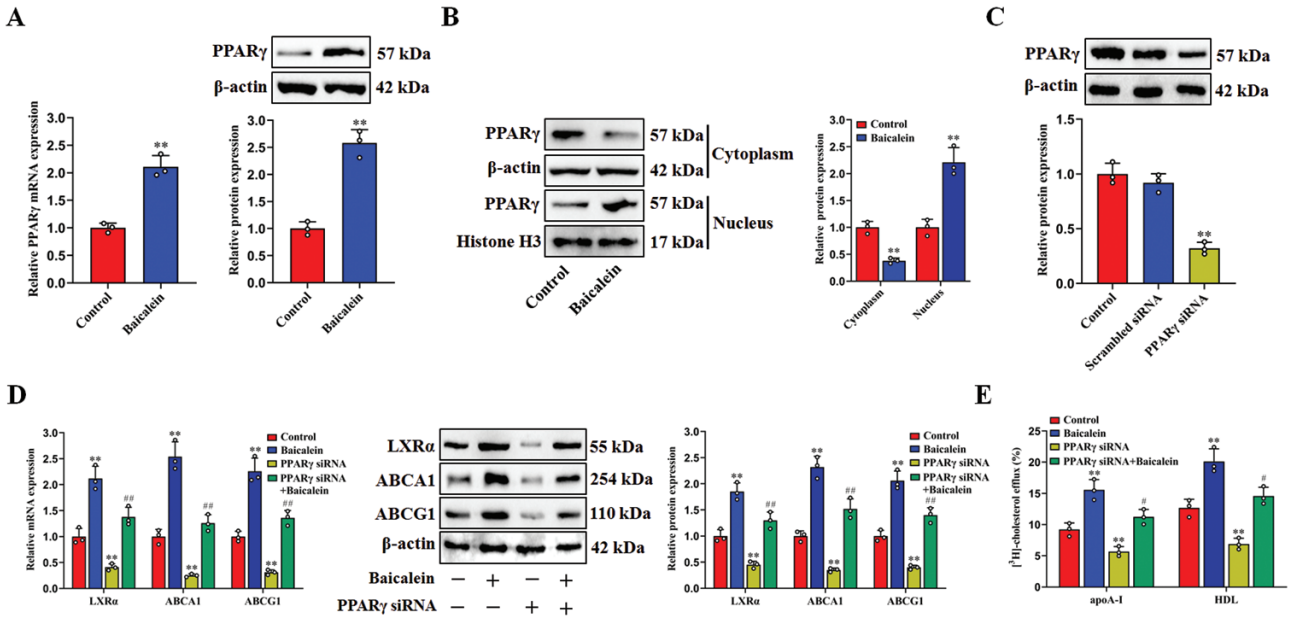


Figure 4: involvement of PPARγ in the baicalein-induced enhancement of LXRα, ABCA1, and ABCG1 expression. (A–B) THP-1 macrophage-derived foam cells were treated with or without 100 μM baicalein for 24 h. (A) PPARγ expression was analyzed by qRT-PCR and western blot. (B) Western blot analysis of PPARγ levels in the cytoplasm and nucleus. (C–E) THP-1 macrophage-derived foam cells were transfected with scrambled siRNA or PPARγ siRNA for 48 h, followed by treatment with or without 100 μM baicalein for 24 h. (C) Detection of PPARγ protein expression using western blot. (D) The expression of LXRα, ABCA1, and ABCG1 was determined by qRT-PCR and western blot. (E) The liquid scintillation counting method was used to measure the efflux of cholesterol onto apoA-I and HDL. The results are shown as the mean ± SD from three independent experiments. $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. baicalein group.

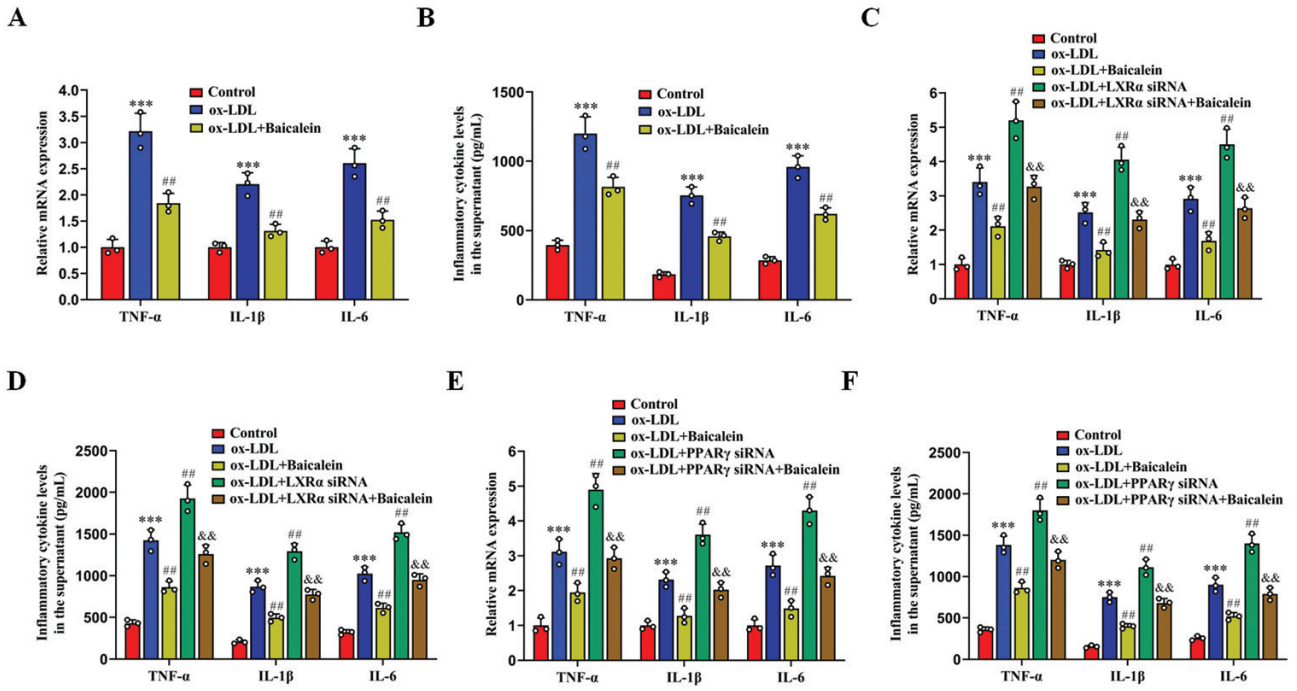


Figure 5: Baicalein inhibits inflammatory response through the PPARγ/LXRα signaling pathway. (A–F) THP-1 macrophages were incubated with or without 50 μg/ml ox-LDL for 48 h, transfected with or without siRNAs against LXRα or PPARγ for 48 h, and then treated with 100 μM baicalein for 24 h. (A, C–E) The mRNA levels of TNF-α, IL-1β, and IL-6 were determined using qRT-PCR. (B, D–F) The cell culture supernatant was collected and then subjected to ELISA for detecting the levels of TNF-α, IL-1β, and IL-6. Data are the mean ± SD from three independent experiments. $^{***}P < 0.001$ vs. control group; $^{\#\#}P < 0.01$ vs. ox-LDL group; $^{\&\&}P < 0.01$ vs. ox-LDL + baicalein group.

cholesterol to lipid-poor/free apoA-I for generating nascent HDL particles, while ABCG1 promotes subsequently continued cholesterol efflux to these particles for further maturation [33]. ABCA1 and ABCG1 export intracellular cholesterol to extracellular acceptors in a synergistic manner [34, 35]. The combined deficiency of these two transporters facilitates foam cell formation and increases atherosclerotic lesion area in mice [36, 37]. CD36 and SR-A are responsible for the internalization of modified lipoproteins. Accumulating evidence has demonstrated that down-regulation of CD36 and SR-A expression alleviates lipid deposition in macrophages [38, 39]. In this study, our results showed that baicalein markedly elevated the mRNA and protein levels of ABCA1 and ABCG1, which was accompanied by increased cholesterol efflux to apoA-I and HDL in THP-1 macrophage-derived foam cells. However, baicalein had no effects on CD36 and SR-A expression and Dil-ox-LDL uptake. These findings provide clear evidence that baicalein inhibits macrophage lipid accumulation by promoting ABCA1- and ABCG1-dependent cholesterol efflux.

LXRα plays a central role in stimulating the transcription of ABCA1 and ABCG1. PPARγ serves as an upstream molecule of LXRα. There is increasing evidence that inactivation of the PPARγ/LXRα pathway down-regulates ABCA1 and ABCG1 expression and attenuates cholesterol efflux in macrophages [40, 41]. The present study demonstrated that administration of baicalein enhanced PPARγ expression and its nuclear translocation in THP-1 macrophage-derived foam cells, in accordance with previous reports [24, 25]. However, Xu et al. reported that treatment with baicalein decreases PPARγ levels and maintains its cytoplasmic retention in a rat model of cerebral ischemia/reperfusion injury [42]. The regulatory effect of baicalein on PPARγ may be cell-specific, and future research is needed to determine what causes the conflicting findings. LXRα was also significantly up-regulated in response to baicalein. Importantly, the stimulatory effect of baicalein on ABCA1 and ABCG1 expression was reversed by siRNA-mediated knockdown of LXRα and PPARγ. Thus, activation of the PPARγ/LXRα signaling pathway is involved in baicalein-induced up-regulation of ABCA1 and ABCG1 expression.

Atherosclerosis is not only a disorder of lipid metabolism dysregulation but also a chronic inflammatory disease [43]. It has been reported that baicalein suppresses inflammatory response in multiple cell types, including mesenchymal stem cells [44], nucleus pulposus cells [45], and pancreatic acinar cells [46]. In the current study, we found that the expression and secretion of IL-1β, IL-6, and TNF-α were significantly decreased in THP-1 macrophage-derived foam cells challenged with baicalein. This finding is consistent with a previous report showing that baicalein suppresses the release of IL-1β, IL-6, and TNF-α in lipopolysaccharide-stimulated RAW264.7 cells [47]. In addition to inhibition of lipid accumulation, the PPARγ/LXRα pathway is involved in the regulation of inflammatory response [22, 23]. Our results revealed that pretreatment with LXRα siRNA and PPARγ siRNA reduced the effects of baicalein on IL-1β, IL-6, and TNF-α expression and secretion, suggesting that this compound activates the PPARγ/LXRα pathway to restrain inflammatory response in THP-1 macrophage-derived foam cells.

Several limitations should be considered in this study. On one hand, there is a lack of animal experiments to explore the influence of baicalein on ABCA1 and ABCG1 expression, reverse cholesterol transport efficiency, plasma lipid profile, and atherosclerotic lesion area. On the other hand, we do not clarify how baicalein up-regulates PPARγ expression. Several lines of evidence have identified PPARγ as a direct target of miR-27 [48, 49]. Baicalein serves as a regulator of many miRNAs [50, 51]. Thus, it is possible that baicalein indirectly regulates PPARγ expression through miR-27 or other miRNAs. Future studies are needed to confirm this possibility.

In summary, our data reveal a favorable role of baicalein in macrophage lipid accumulation and inflammatory response. Mechanistically, baicalein activates the PPARγ/LXRα pathway to enhance ABCA1- and ABCG1-dependent cholesterol efflux and inhibit pro-inflammatory cytokine release. These findings extend our understanding for the anti-atherogenic action of baicalein, and further support the notion that baicalein may be a promising drug candidate for therapeutic intervention of atherosclerotic cardiovascular disease.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

Acknowledgements

The authors gratefully acknowledge the financial support from the Scientific Research Program of Higher Education of Hainan Province (Hnky2020-43) and the Hainan Provincial Natural Science Foundation of China (820RC767).

Conflicts of interest

The authors declare they do not have anything to disclose regarding conflicts of interest with respect to this manuscript.

References

1. Yu XH, Fu YC, Zhang DW, Yin K, Tang CK. Foam cells in atherosclerosis. *Clin Chim Acta* 2013, 424, 245–52. doi:10.1016/j.cca.2013.06.006.
2. Wang G, Gao JH, He LH, Yu XH, Zhao ZW, Zou J, et al. Fargesin alleviates atherosclerosis by promoting reverse cholesterol transport and reducing inflammatory response. *Biochim Biophys Acta Mol Cell Biol Lipids* 2020, 1865, 158633. doi:10.1016/j.bbalip.2020.158633.
3. Gui YZ, Yan H, Gao F, Xi C, Li HH, Wang YP. Betulin attenuates atherosclerosis in apoE mice by up-regulating ABCA1 and ABCG1. *Acta Pharmacol Sin* 2016, 37, 1337–48. doi:10.1038/aps.2016.46.
4. Wang Z, Shi H, Zhao H, Dong Z, Zhao B, Weng X, et al. Naoxintong retards atherosclerosis by inhibiting foam cell formation through activating Pparα pathway. *Curr Mol Med* 2018, 18, 698–710.
5. Persson J, Nilsson J, Lindholm MW. Interleukin-1beta and tumour necrosis factor-alpha impede neutral lipid turnover in macrophage-derived foam cells. *BMC Immunol* 2008, 9, 70.
6. Lavin Plaza B, Phinikaridou A, Andia ME, Potter M, Lorrio S, Rashid I, et al. Sustained focal vascular inflammation accelerates atherosclerosis in remote arteries. *Arterioscler Thromb Vasc Biol* 2020, 40, 2159–70.
7. Fruchart JC, Davignon J, Hermans MP, Al-Rubeaan K, Amarenco P, Assmann G, et al. Residual macrovascular risk in 2013: what have we learned? *Cardiovasc Diabetol* 2014, 13, 26.

8. Sirimarco G, Labreuche J, Bruckert E, Goldstein LB, Fox KM, Rothwell PM, et al. Atherogenic dyslipidemia and residual cardiovascular risk in statin-treated patients. *Stroke* 2014, 45, 1429–36.
9. Wang CZ, Zhang CF, Luo Y, Yao H, Yu C, Chen L, et al. Baicalein, an enteric microbial metabolite, suppresses gut inflammation and cancer progression in ApcMin/+ mice. *Clin Transl Oncol* 2020, 22, 1013–22.
10. Dong Y, Xing Y, Sun J, Sun W, Xu Y, Quan C. Baicalein alleviates liver oxidative stress and apoptosis induced by high-level glucose through the activation of the PERK/Nrf2 signaling pathway. *Molecules* 2020, 25, 599.
11. Oo A, Teoh BT, Sam SS, Bakar SA, Zandi K. Baicalein and baicalin as Zika virus inhibitors. *Arch Virol* 2019, 164, 585–93.
12. Song L, Yang H, Wang HX, Tian C, Liu Y, Zeng XJ, et al. Inhibition of 12/15 lipoxygenase by baicalein reduces myocardial ischemia/reperfusion injury via modulation of multiple signaling pathways. *Apoptosis* 2014, 19, 567–80.
13. Shi R, Wei Z, Zhu D, Fu N, Wang C, Yin S, et al. Baicalein attenuates monocrotaline-induced pulmonary arterial hypertension by inhibiting vascular remodeling in rats. *Pulm Pharmacol Ther* 2018, 48, 124–35.
14. Zhao F, Fu L, Yang W, Dong Y, Yang J, Sun S, et al. Cardioprotective effects of baicalein on heart failure via modulation of Ca(2+) handling proteins in vivo and in vitro. *Life Sci* 2016, 145, 213–23.
15. Chen H, Xu Y, Wang J, Zhao W, Ruan H. Baicalin ameliorates isoproterenol-induced acute myocardial infarction through iNOS, inflammation and oxidative stress in rat. *Int J Clin Exp Pathol* 2015, 8, 10139–47.
16. Chan SH, Hung CH, Shih JY, Chu PM, Cheng YH, Tsai YJ, et al. Baicalein is an available anti-atherosclerotic compound through modulation of nitric oxide-related mechanism under oxLDL exposure. *Oncotarget* 2016, 7, 42881–91.
17. Zhang Y, Ma G, Li C, Cao Z, Qie F, Xu X. Baicalein inhibits VSMCs proliferation via regulating LncRNAAK021954 gene expression. *Int J Clin Exp Med* 2015, 8, 22129–38.
18. Im SS, Osborne TF. Liver x receptors in atherosclerosis and inflammation. *Circ Res* 2011, 108, 996–1001.
19. Wang H, Yang Y, Sun X, Tian F, Guo S, Wang W, et al. Sonodynamic therapy-induced foam cells apoptosis activates the phagocytic PPARγ-LXRα-ABCA1/ABCG1 pathway and promotes cholesterol efflux in advanced plaque. *Theranostics* 2018, 8, 4969–84.
20. Jiang T, Ren K, Chen Q, Li H, Yao R, Hu H, et al. Leonurine prevents atherosclerosis via promoting the expression of ABCA1 and ABCG1 in a Pparγ/Lxrα signaling pathway-dependent manner. *Cell Physiol Biochem* 2017, 43, 1703–17.
21. Hu YW, Ma X, Huang JL, Mao XR, Yang JY, Zhao JY, et al. Dihydrocapsaicin attenuates plaque formation through a PPARγ/LXRα pathway in apoE(–/–) mice fed a high-fat/high-cholesterol diet. *PLoS One* 2013, 8, e66876.
22. Tang J, Luo K, Li Y, Chen Q, Tang D, Wang D, et al. Capsaicin attenuates LPS-induced inflammatory cytokine production by upregulation of LXRα. *Int Immunopharmacol* 2015, 28, 264–9.
23. Singh V, Rana M, Jain M, Singh N, Naqvi A, Malasoni R, et al. Curcuma oil attenuates accelerated atherosclerosis and macrophage foam-cell formation by modulating genes involved in plaque stability, lipid homeostasis and inflammation. *Br J Nutr* 2015, 113, 100–13.
24. Kim DH, Hossain MA, Kang YJ, Jang JY, Lee YJ, Im E, et al. Baicalein, an active component of *Scutellaria baicalensis* Georgi, induces apoptosis in human colon cancer cells and prevents AOM/DSS-induced colon cancer in mice. *Int J Oncol* 2013, 43, 1652–8.
25. Cao H, Li S, Xie R, Xu N, Qian Y, Chen H, et al. Exploring the mechanism of Dangguiliuhuang decoction against hepatic fibrosis by network pharmacology and experimental validation. *Front Pharmacol* 2018, 9, 187.
26. Varghese JF, Patel R. Yadav UCS. Sterol regulatory element binding protein (SREBP) -1 mediates oxidized low-density lipoprotein (oxLDL) induced macrophage foam cell formation through NLRP3 inflammasome activation. *Cell Signal* 2019, 53, 316–26.
27. Kumar A, Gupta P, Rana M, Chandra T, Dikshit M, Barthwal MK. Role of pyruvate kinase M2 in oxidized LDL-induced macrophage foam cell formation and inflammation. *J Lipid Res* 2020, 61, 351–64.
28. Chistiakov DA, Melnichenko AA, Myasoedova VA, Grechko AV, Orekhov AN. Mechanisms of foam cell formation in atherosclerosis. *J Mol Med (Berl)* 2017, 95, 1153–65.
29. Yin R, Zhu X, Wang J, Yang S, Ma A, Xiao Q, et al. MicroRNA-155 promotes the ox-LDL-induced activation of NLRP3 inflammasomes via the ERK1/2 pathway in THP-1 macrophages and aggravates atherosclerosis in ApoE-/- mice. *Ann Palliat Med* 2019, 8, 676–89.
30. Mangal P, Khare P, Jagtap S, Bishnoi M, Kondepudi KK, Bhutani KK. Screening of six Ayurvedic medicinal plants for anti-obesity potential: An investigation on bioactive constituents from Oroxylum indicum (L.) Kurz bark. *J Ethnopharmacol* 2017, 197, 138–46.
31. Yin H, Huang L, Ouyang T, Chen L. Baicalein improves liver inflammation in diabetic db/db mice by regulating HMGB1/TLR4/NF-κB signaling pathway. *Int Immunopharmacol* 2018, 55, 55–62.
32. He XW, Yu D, Li WL, Zheng Z, Lv CL, Li C, et al. Anti-atherosclerotic potential of baicalin mediated by promoting cholesterol efflux from macrophages via the PPARγ-LXRα-ABCA1/ABCG1 pathway. *Biomed Pharmacother* 2016, 83, 257–64.
33. Yu XH, Zhang DW, Zheng XL, Tang CK. Cholesterol transport system: An integrated cholesterol transport model involved in atherosclerosis. *Prog Lipid Res* 2019, 73, 65–91.
34. Gelissen IC, Harris M, Rye KA, Quinn C, Brown AJ, Kockx M, et al. ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I. *Arterioscler Thromb Vasc Biol* 2006, 26, 534–40.
35. Vaughan AM, Oram JF. ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL. *J Lipid Res* 2006, 47, 2433–43.
36. Yvan-Charvet L, Ranalletta M, Wang N, Han S, Terasaka N, Li R, et al. Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. *J Clin Invest* 2007, 117, 3900–8.
37. Westerterp M, Tsuchiya K, Tattersall IW, Fotakis P, Bochem AE, Molusky MM, et al. Deficiency of ATP-binding cassette transporters A1 and G1 in endothelial cells accelerates atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 2016, 36, 1328–37.
38. Liu D, Wang X, Zhang M, Tian J, Liu M, Jin T, et al. WISP1 alleviates lipid deposition in macrophages via the PPARγ/CD36 pathway in the plaque formation of atherosclerosis. *J Cell Mol Med* 2020, 24, 11729–41.
39. Yang Y, Lian YT, Huang SY, Yang Y, Cheng LX, Liu K. GABA and topiramate inhibit the formation of human macrophage-derived foam cells by modulating cholesterol-metabolism-associated molecules. *Cell Physiol Biochem* 2014, 33, 1117–29.
40. Xu Z, Dong A, Feng Z, Li J. Interleukin-32 promotes lipid accumulation through inhibition of cholesterol efflux. *Exp Ther Med* 2017, 14, 947–52.
41. Bogachev O, Majdalawieh A, Pan X, Zhang L, Ro HS. Adipocyte enhancer-binding protein 1 (AEBP1) (a novel macrophage proinflammatory mediator) overexpression promotes and ablation attenuates atherosclerosis in ApoE (–/–) and LDLR (–/–) mice. *Mol Med* 2011, 17, 1056–64.
42. Xu YW, Sun L, Liang H, Sun GM. Cheng Y. 12/15-Lipoxygenase inhibitor baicalein suppresses PPAR gamma expression and nuclear translocation induced by cerebral ischemia/reperfusion. *Brain Res* 2010, 1307, 149–57.
43. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med* 1999, 340, 115–26.
44. Zhu L, Liu YJ, Shen H, Gu PQ, Zhang L. Astragalus and baicalein regulate inflammation of mesenchymal stem cells (MSCs) by the mitogen-activated protein kinase (MAPK)/ERK pathway. *Med Sci Monit* 2017, 23, 3209–16.
45. Jin H, Wang Q, Wu J, Han X, Qian T, Zhang Z, et al. Baicalein inhibits the IL-1β-induced inflammatory response in nucleus

pulposus cells and attenuates disc degeneration in vivo. *Inflammation* 2019, 42, 1032–44.
46. Li J, Zhou R, Bie BB, Huang N, Guo Y, Chen HY, et al. Emodin and baicalein inhibit sodium taurocholate-induced vacuole formation in pancreatic acinar cells. *World J Gastroenterol* 2018, 24, 35–45.
47. Qi Z, Yin F, Lu L, Shen L, Qi S, Lan L, et al. Baicalein reduces lipopolysaccharide-induced inflammation via suppressing JAK/STATs activation and ROS production. *Inflamm Res* 2013, 62, 845–55.
48. Lin J, Maimaitiyiming A, Chen S, Xiao M, Xian Z. Hypoxia-induced miR-27 and miR-195 regulate ATP consumption, viability, and metabolism of rat cardiomyocytes by targeting PPARγ and FASN expression. *Aging (Albany NY)* 2021, 13, 10158–74.
49. Sun L, Trajkovski M. MiR-27 orchestrates the transcriptional regulation of brown adipogenesis. *Metabolism* 2014, 63, 272–82.
50. Cui X, Sun X, Lu F, Jiang X. Baicalein represses TGF-β1-induced fibroblast differentiation through the inhibition of miR-21. *Toxicol Appl Pharmacol* 2018, 358, 35–42.
51. Bie B, Sun J, Li J, Guo Y, Jiang W, Huang C, et al. Baicalein, a natural anti-cancer compound, alters microRNA expression profiles in bel-7402 human hepatocellular carcinoma cells. *Cell Physiol Biochem* 2017, 41, 1519–31.