

Berberine promotes the development of atherosclerosis and foam cell formation by inducing scavenger receptor A expression in macrophage

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Berberine is identified to lower the serum cholesterol level in human and hamster through the induction of low density lipoproteins (LDL) receptor in hepatic cells. To evaluate its potential in preventing atherosclerosis, the effect of berberine on atherosclerosis development in apolipoprotein E-deficient (apoE^{-/-}) mice was investigated. In apoE^{-/-} mice, berberine induced *in vivo* foam cell formation and promoted atherosclerosis development. The foam cell formation induced by berberine was also observed in mouse RAW264.7 cells, as well as in mouse and human primary macrophages. By inducing scavenger receptor A (SR-A) expression in macrophages, berberine increased the uptake of modified LDL (DiO-Ac-LDL). Berberine-induced SR-A expression was also observed in macrophage foam cells *in vivo* and in the cells at atherosclerotic lesion. Analysis in RAW264.7 cells indicated that berberine induced SR-A expression by suppressing PTEN expression, which led to sustained Akt activation. Our results suggest that to evaluate the potential of a cholesterol-reducing compound in alleviating atherosclerosis, its effect on the cells involved in atherosclerosis development, such as macrophages, should also be considered. Promotion of foam cell formation could counter-balance the beneficial effect of lowering serum cholesterol.

Keywords: berberine, scavenger receptor A, macrophage foam cell, atherosclerosis, PI3-kinase-PTEN
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Introduction

Reduction of serum cholesterol level has been proven as an effective way to prevent atherosclerosis development [1]. The success of statin drugs and ezetimibe in preventing atherosclerosis by reducing serum low density lipoproteins (LDL) cholesterol level has instigated the search for cholesterol-controlling products [1-4]. Recently, berberine, a natural alkaloid isolated from Huanglian (*Coptis chinensis*), is reported to lower serum cholesterol level in human and hamster [5]. The effect of berberine on lowering serum LDL cholesterol is mediated by stabilizing LDL receptor mRNA in the liver cells [5, 6]. This

represents a different mechanism from statins, which induce LDL receptor expression through inactivating 3-hydroxy-3-methylglutaryl coenzyme A reductase [2].

Berberine has been used as a non-prescription drug to treat diarrhea and gastroenteritis in China since 1950s. It is reported to have anti-diabetic effects in insulin-responsive cells and animal models [7]. In 3T3-L1 adipocytes and L6 myotubes, berberine activates AMP-activated protein kinase and induces GLUT4 translocation [7]. During 3T3-L1 adipocyte differentiation induction, berberine reduces the lipid accumulation in 3T3-L1 cells by inactivating PPAR γ [7, 8]. These beneficial metabolic effects of berberine and its history as a non-prescription drug in China suggest that berberine has the potential to be a drug for treating hyperlipidemia, hyperglycemia and obesity.

Atherosclerosis is a chronic inflammation condition resulting from the interaction between lipoproteins, monocyte-derived macrophages, T lymphocytes and other cellular elements in the arterial wall [9-11]. During

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atherosclerosis development, the attraction of circulating monocytes by modified LDL into subendothelial arterial space and their differentiation into macrophages are the initial events [12-15]. In the subendothelial arterial space, the uptake of modified LDL by macrophages leads to the formation of foam cells in which a large amount of cholesteryl ester droplets are accumulated [9, 15-17]. The release of cholesteryl lipids from apoptotic foam cell forms the initial plaques in subendothelial arterial space. The role of foam cells as the major culprit in atherosclerosis has been further demonstrated by the resistance to atherosclerosis in mice that lack macrophages [18, 19].

In macrophage, the uptake of modified LDL by scavenger receptors induces the formation of foam cell [20-23]. Besides hypercholesterolemia, other factors such as LDL oxidation and increased scavenger receptors can also disrupt the cholesterol homeostasis in macrophage, induce foam cell formation and cause atherosclerosis. As berberine targets multiple types of cells such as hepatocytes, adipocytes and muscle cells [5-8], it is important to ascertain whether berberine has any effect on monocytes/macrophages, especially on foam cell formation. In order to evaluate the potential of berberine as a cholesterol-controlling drug for preventing atherosclerosis, its effect on atherosclerosis development needs to be more carefully investigated. In this report, we show that in apolipoprotein E-deficient (apoE^{-/-}) mice, berberine promotes atherosclerosis development. The promotion of atherosclerosis resulted from berberine-induced *in vivo* foam cell formation. Using RAW264.7 cells, which can be induced by oxidized LDL (oxLDL) to form foam cells [24], we have identified that berberine induces the expression of scavenger receptor A (SR-A) through modulating the PI3-kinase signaling pathway. Specifically, by inactivating PPAR γ , berberine inhibits PTEN expression and thus enhances PI3-kinase signaling.

Results

Berberine promotes atherosclerosis development in apoE^{-/-} mice by inducing in vivo foam cell formation

ApoE^{-/-} mouse is a well-established animal model for studying atherosclerosis [25-27]. Because berberine is poorly absorbed if taken orally [28], we administrated berberine to mice by intraperitoneal injection. On average, berberine-treated mice took less food and gained less weight than the control mice (Figure 1A). After a 15-week berberine treatment, there was no significant change in the total serum triglycerides, LDL cholesterol or HDL cholesterol levels (Figure 1B). However, more atherosclerotic lesions were developed in berberine-treated mice (Figure 1C). The average size of atherosclerotic

lesions in berberine-treated mice was about 50% larger than that in control mice (Figure 1D).

Because there was no change in serum cholesterol in berberine-treated mice, the berberine-promoted atherosclerosis would likely have resulted from other factors than serum cholesterol (Figure 1B). As foam cell is the major culprit in atherosclerosis [18, 19], peritoneal macrophages were collected from berberine-treated apoE^{-/-} mice and analyzed for foam cell formation. The peritoneal macrophages collected from control apoE^{-/-} mice exhibited the typical macrophage morphology and contained little amount of lipid droplets (Figure 1E). In contrast, most macrophages collected from berberine-treated apoE^{-/-} mice contained massive amount of lipid droplets and the cellular cholesterol content was increased by several folds (Figure 1E and 1F).

The promotion of *in vivo* foam cell formation by berberine was confirmed by *in vitro* experiments with mouse primary macrophages. In normal culture medium with 10% fetal bovine serum (FBS), no foam cell was induced from the cultured mouse peritoneal macrophages (Figure 2A and 2B). When the cultured primary macrophages were treated with berberine, foam cell formation was induced and cellular cholesterol level was also increased (Figure 2A and 2B). The induction of foam cell formation by berberine in human primary macrophages was also observed (Figure 2C). Although the effect of berberine on serum cholesterol level in apoE^{-/-} mouse was different from its reported effect in human (Figure 1B) [5], the promotion of foam cell formation was similar in cultured primary macrophages of both mouse and human (Figure 2).

Berberine promotes foam cell formation by inducing SR-A

In comparison to primary cells, the *in vitro* cultured cell line is usually more feasible for performing mechanistic studies. Mouse RAW264.7 cells are a macrophage-like cell line and can be induced to form foam cells in the presence of oxLDL (Supplementary information, Figure S1) [24]. It could also be induced to form foam cells by berberine without the addition of oxLDL (Figure 2A). The foam cell formation and cholesterol accumulation induced by berberine in RAW264.7 cells were very similar to that in mouse primary macrophages (Figure 2A and 2B). Thus RAW264.7 cells appear to be a good model for studying the mechanism of berberine induction of foam cell formation (Supplementary information, Figure S1).

SR-A and CD36 are two most important scavenger receptors for the uptake of modified LDL, which promotes foam cell formation in macrophages [22]. Berberine

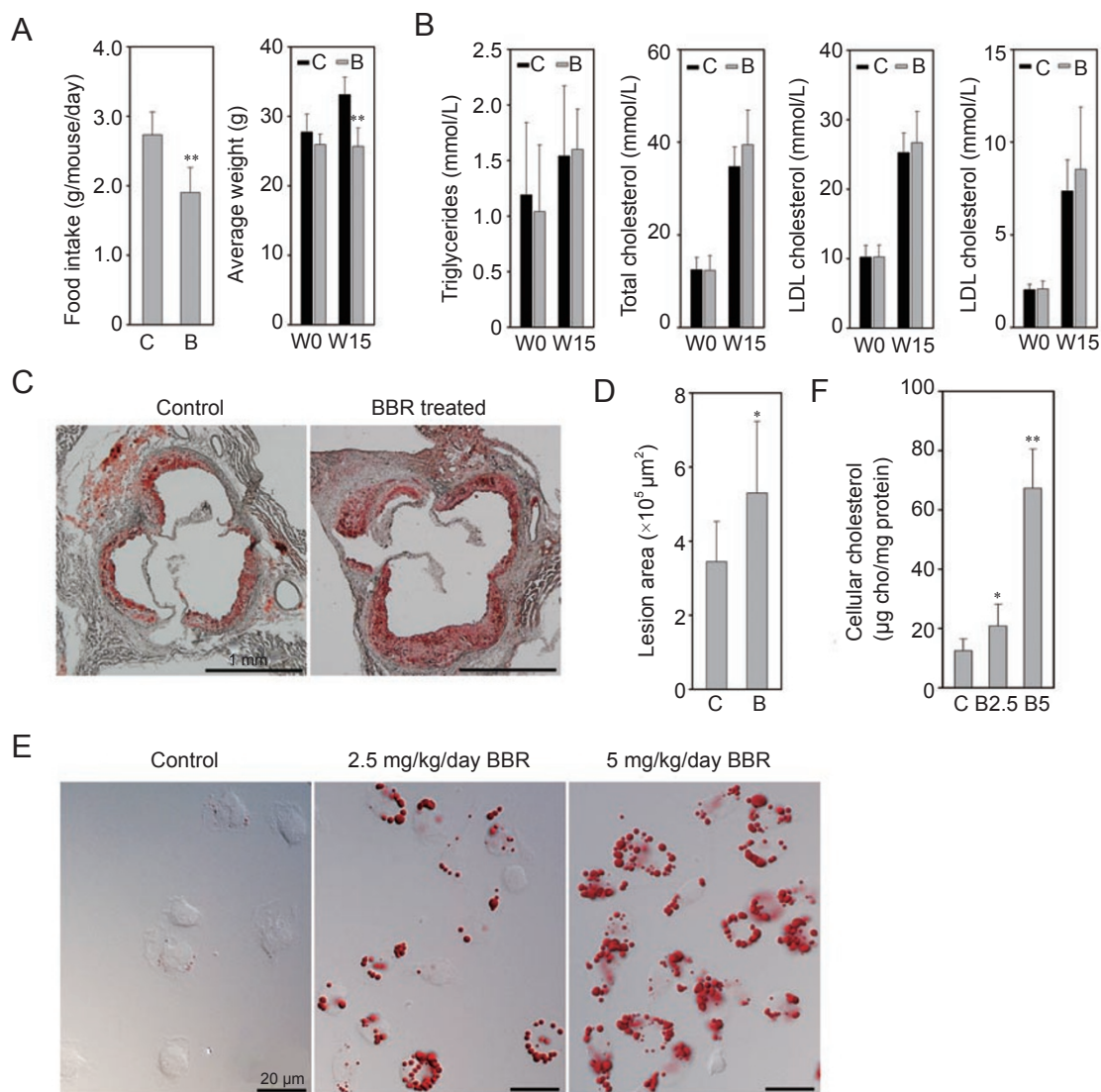


Figure 1 Berberine promotes atherosclerosis development by inducing *in vivo* foam cell formation in apoE^{-/-} mice. **(A)** Food intake and body weight of mice ($n = 10$ per group). The daily food intake was measured every other week. The results are the average of eight measurements. The body weight is the average weight of 10 mice at the beginning on Western diet (W0) and after 15-week experiment (W15). C, mice with vehicle treatment; B, mice with berberine treatment (5 mg/kg/day). **(B)** Serum lipids. The labels are the same as in panel A. The results are the average of 10 mice. **(C)** Atherosclerotic lesions in the aortic root after 15-week experiment. Cryosection of aortic sinus was stained with Oil-red-O. Representative photographs are shown. **(D)** The average lesion size. For each mouse, the Oil-red-O-stained areas were measured in four sections of the aortic sinus and these were averaged to obtain the lesion size of one mouse. For each group, 10 mice were averaged. * $P < 0.05$. **(E)** *In vivo* foam cell formation in berberine-treated mice ($n=6$ per group). Oil-red-O staining of peritoneal macrophages isolated from mice treated with berberine for 2 weeks (2.5 mg/kg/day BBR and 5 mg/kg/day BBR) or not (Control). Representative photographs are shown. **(F)** Cholesterol content of peritoneal macrophages. The results are the average of six mice. B2.5, 2.5 mg/kg/day berberine; B5, 5 mg/kg/day berberine. * $P < 0.05$, ** $P < 0.01$.

treatment increased the levels of SR-A on RAW264.7 cell surface, but not of CD36 (Figure 3A and 3B). It also induced DiO-Ac-LDL uptake in RAW264.7 cells (Figure 3C). This berberine-induced DiO-Ac-LDL uptake clearly resulted from SR-A increase, as it was specifically in-

hibited by antibody against SR-A (Figure 3D). In addition, berberine did not affect the cholesterol efflux from RAW264.7 cells (Figure 3E). These observations suggest that berberine-induced SR-A is the primary cause for the increased foam cell formation.

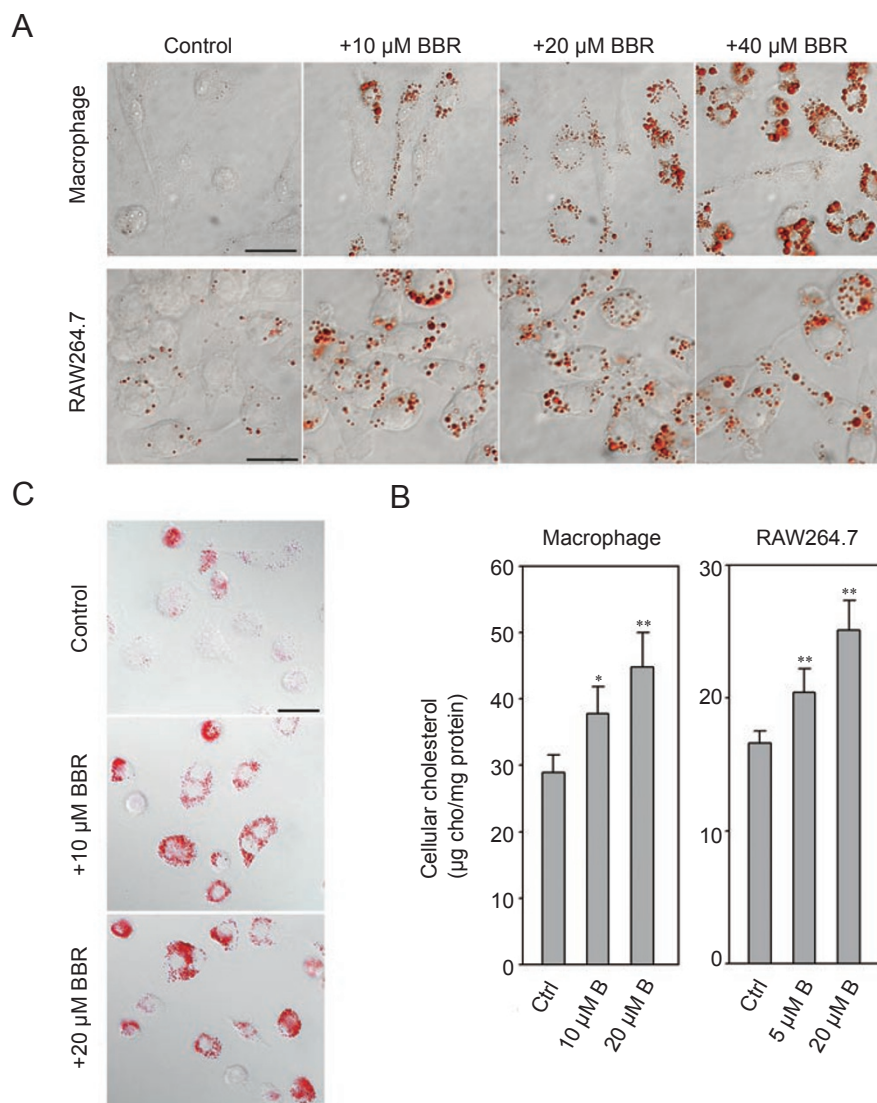


Figure 2 Berberine induces *in vitro* foam cell formation. Bar = 10 µm. **(A)** Berberine induced foam cell formation in cultured mouse peritoneal macrophage (macrophage) and RAW264.7 cell (RAW264.7). +10 µM BBR, +20 µM BBR and +40 µM BBR indicate berberine concentration. Control, no berberine treatment. **(B)** Cholesterol of mouse peritoneal macrophage and RAW264.7 cell. The results are the average of three independent experiments. * $P < 0.05$, ** $P < 0.01$. 5 µM B, 10 µM B and 20 µM B indicate berberine concentration. **(C)** Berberine induced foam cell formation in human primary macrophage.

SR-A was expressed at a relatively constant level in RAW264.7 cells (Figure 3F). However, it was greatly induced by berberine treatment (Figure 3A and 3F). The analysis of SR-A mRNA and its gene promoter activity showed that berberine induced SR-A by increasing gene transcription (Figure 3G and 3H). To identify the signal molecules involved in berberine-induced SR-A transcription, the effect of PI3-kinase inhibitor (LY294002) or MEK inhibitors (PD98059 and U0126) on berberine-induced foam cell formation was analyzed. Only LY294002, but not PD98059 or U0126, inhibited

berberine-induced foam cell formation (Supplementary information, Figure S2). Interestingly, LY294002 did not inhibit the basal SR-A expression, but inhibited berberine-induced SR-A expression (Figure 3H and 3I).

To ascertain whether berberine promotes atherosclerosis development in apoE^{-/-} mouse by inducing SR-A, atherosclerotic lesions were analyzed for SR-A expression. As shown in Figure 4A, SR-A expression in the cells of atherosclerotic lesions was greatly enhanced in berberine-treated apoE^{-/-} mice. Western blot of aorta samples also revealed the increased SR-A expression in

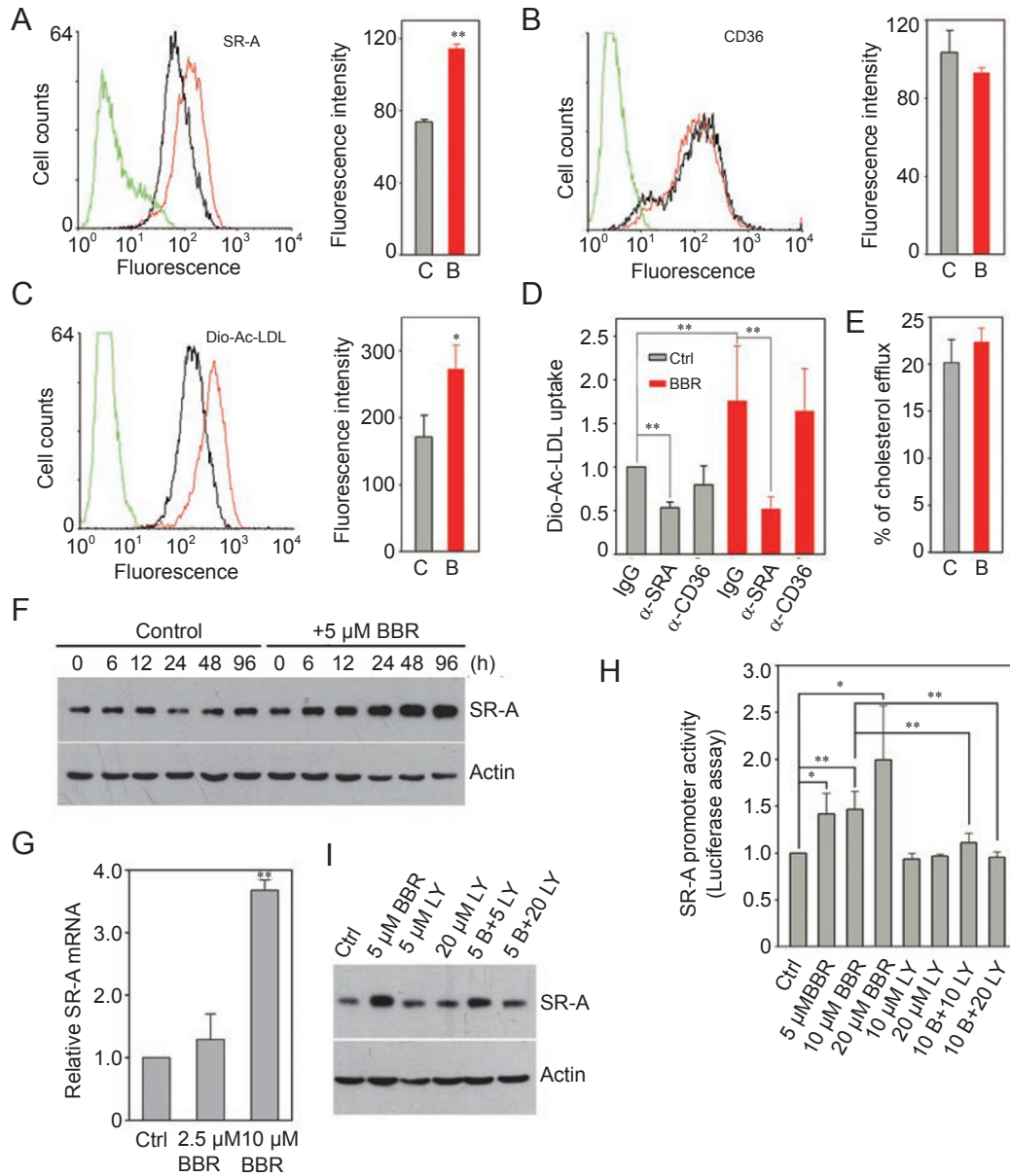


Figure 3 Berberine specifically induces scavenger receptor A expression. * $P < 0.05$, ** $P < 0.01$. **(A)** Cell surface SR-A. The flow cytometry graph is the fluorescence labeling by anti-SR-A antibody in RAW264.7 cells treated with (Red line) or without (Gray line) 10 μM berberine. The green line is the blank IgG. The bar graph is the average result of three experiments. C, control; B, berberine-treated cell. **(B)** Cell surface CD36. The labels are the same as in panel A. **(C)** DiO-Ac-LDL uptake. Gray line, control cell; Red line, berberine-treated cell; Green line, without adding DiO-Ac-LDL. **(D)** Inhibition of DiO-Ac-LDL uptake by anti-SR-A antibody. The gray bars are DiO-Ac-LDL uptake in control RAW264.7 cells blocked with blank IgG (IgG), anti-SR-A antibody (α-SRA) or anti-CD36 antibody (α-CD36). The red bars are DiO-Ac-LDL uptake in berberine-treated RAW264.7 cells blocked with antibody. **(E)** Cholesterol efflux. RAW264.7 cells were labeled with ³H-labeled cholesterol (Perkin-Elmer) and cholesterol efflux was measured in the presence or absence of 5 μM berberine as described by de la Llera Moya *et al.* [47]. The results are the average of three experiments. **(F)** Time course of berberine induced SR-A expression. SR-A in RAW264.7 cell treated with or without (Control) 5 μM berberine (5 μM BBR) for the indicated times (0, 6, 12, 24, 48 and 96 h) was detected by western blot. **(G)** Real-time PCR of SR-A mRNA in RAW264.7 cell treated with or without (Ctrl) berberine for 48 h (2.5 μM BBR or 10 μM BBR). The results are the average of four experiments. **(H)** SR-A promoter activity. The luciferase analysis was carried out in 293T cells transfected with SR-A promoter-luciferase reporter plasmid and treated with berberine (5 μM BBR, 10 μM BBR and 20 μM BBR), LY294002 (10 μM LY and 20 μM LY) or both (10 B+10 LY and 10 B+20 LY) for 24 h. The results are the average of four experiments. **(I)** Inhibition of berberine-induced SR-A expression by LY294002. SR-A in RAW264.7 cell treated with berberine (5 μM BBR), LY294002 (5 μM LY or 20 μM LY) or both (5 B+5 LY and 5 B+20 LY) for 48 h was detected by western blot.

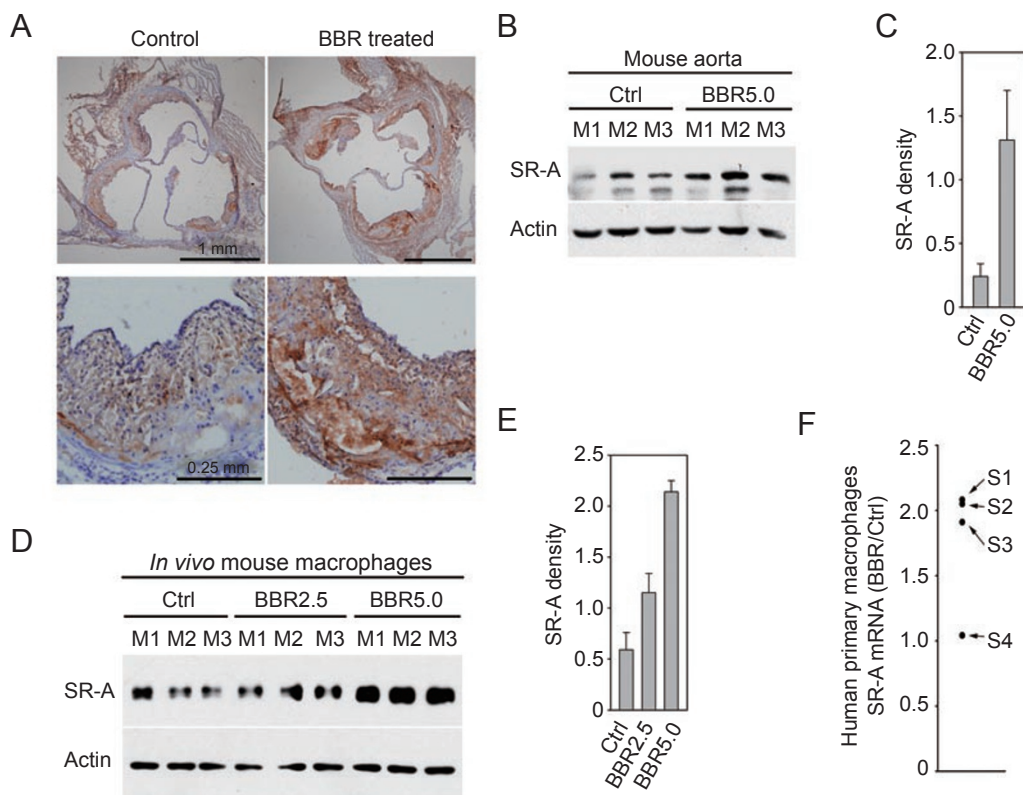


Figure 4 Berberine induces SR-A expression *in vivo*. **(A)** SR-A expression in cells at atherosclerotic lesion. Sections of aortic sinus were stained with anti-SR-A antibody (brown) and the nuclei were counter-stained with hematoxylin (blue). Representative photographs are shown. Control, control apoE^{-/-} mice; BBR treated, apoE^{-/-} mice treated with berberine for 15 weeks. **(B)** Western blot for SR-A in aorta samples. BBR5.0, apoE^{-/-} mice treated with 5 mg/kg/day berberine for 4 weeks; M1, M2 and M3, individual mice. **(C)** Densitometer scanning of western blot in panel B. **(D)** Western blot for SR-A in peritoneal macrophages isolated from apoE^{-/-} mice treated with or without (Ctrl) 2.5 mg/kg/day or 5 mg/kg/day berberine for 2 weeks (BBR2.5 and BBR5.0). **(E)** Densitometer scanning of western blot in panel D. **(F)** SR-A expression in human primary macrophages. Human primary macrophages of four volunteers (S1-4) were treated with 10 μ mol/l berberine or not for 24 h. SR-A mRNA was determined by real-time RT-PCR. The ratio of berberine treatment to control was plotted.

berberine-treated apoE^{-/-} mice (Figure 4B and 4C). In peritoneal macrophages obtained from berberine-treated apoE^{-/-} mice, SR-A was increased in a dose-dependent manner (Figure 4D and 4E). Human primary macrophage samples from three of the four volunteers also exhibited berberine-induced SR-A expression (Figure 4F).

Berberine induces SR-A expression by activating PI3-kinase signaling

LY294002, which inhibited berberine-induced SR-A expression and foam cell formation (Figure 3H, 3I and Supplementary information, Figure S2), is a widely used PI3-kinase inhibitor. The inhibitory effect of LY294002 on foam cell formation could be reversed by the addition of 3-phosphorylated phosphatidylinositol (dipalmitoyl-L-alpha-phosphatidylinositol-3, 4, 5-trisphosphate) (Figure 5A). The PI3-kinase signal cascade includes PI3-

kinase that catalyzes the formation of 3-phosphorylated phosphatidylinositols and PTEN that dephosphorylates 3-phosphorylated phosphatidylinositol [29]. By blocking PI3-kinase activity, LY294002 inhibited Akt-1 activation (Supplementary information, Figure S2) [30]. Berberine treatment decreased PTEN expression without affecting PI3-kinase (p85 and p110 subunits) (Figure 5B and 5C). Decrease of PTEN could increase the stability of 3-phosphorylated phosphatidylinositols, which leads to sustained Akt-1 activation (Figure 5D).

Berberine reduced PTEN mRNA levels (Figure 5E). The activation of Akt only after prolonged berberine treatment indicated a relatively slow effect on PTEN expression, likely through transcription regulation (Figure 5D and Supplementary information, Figure S2). It is reported that berberine inhibits PPAR γ activity by increasing PPAR γ phosphorylation [7, 8] and PTEN transcrip-

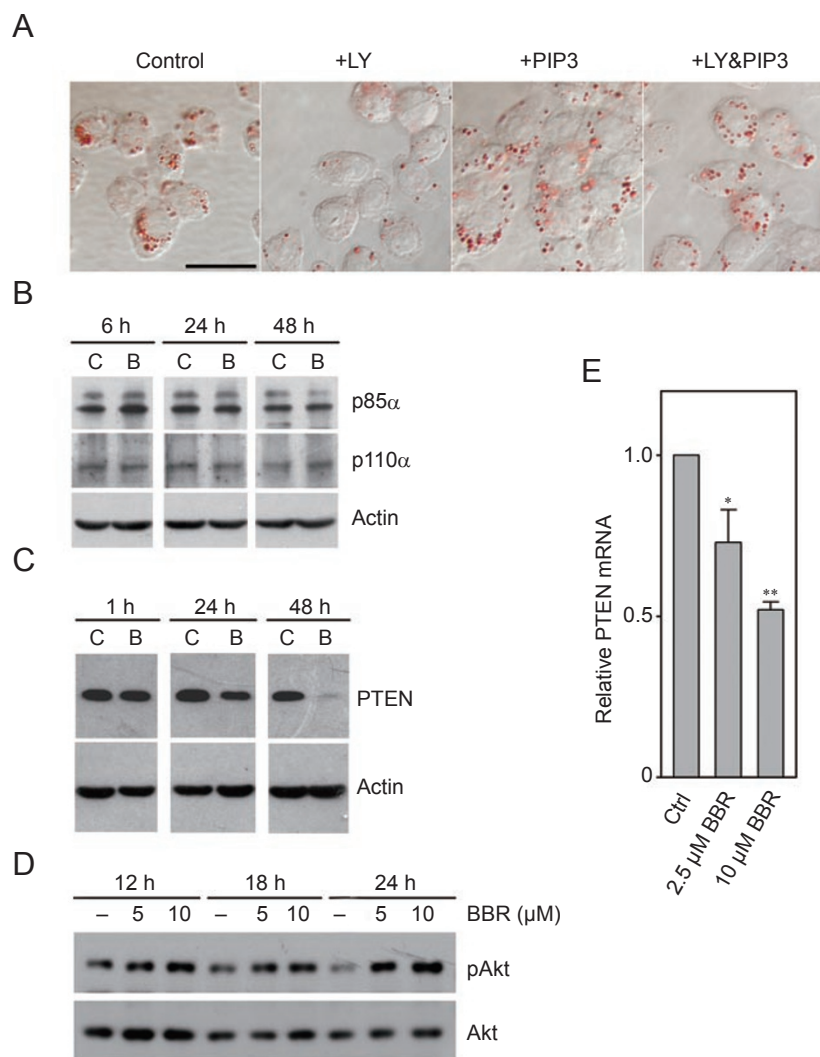


Figure 5 Berberine reduces PTEN expression. **(A)** Oil-red-O staining of RAW264.7 cells treated with 10 μ M LY294002 (+LY), 5 μ M dipalmitoyl-L-alpha-phosphatidylinositol-3, 4, 5-trisphosphate (+PIP3), or both (+LY & PIP3) for 4 days. Bar = 10 μ m. Control, RAW264.7 cells 4 days after cell-cell contact. Cells were replated and then stained. **(B)** p85 subunit and p110 subunit of PI3-kinase in RAW264.7 cells treated with 5 μ M berberine (B) for the indicated times (6 h, 24 h and 48 h) or not (C). **(C)** PTEN in RAW264.7 cells treated with berberine. The labels are the same as in panel B. **(D)** Akt-1 phosphorylation in berberine-treated RAW264.7 cell. **(E)** PTEN mRNA in RAW264.7 cells treated with or without (Ctrl) berberine for 48 h (2.5 μ M BBR and 10 μ M BBR) was measured by real-time RT-PCR. The results are the average of three experiments. * $P < 0.05$, ** $P < 0.01$.

tion is regulated by PPAR γ [31, 32]. The inhibition of PPAR γ by berberine was clearly illustrated by berberine-mediated inhibition of 3T3-L1 adipocyte differentiation, as PPAR γ is one of the most important transcription factors in adipocyte differentiation (Figure 6A) [7, 8, 33, 34]. In RAW264.7 cells, berberine inhibited PTEN expression induced by troglitazone, an agonist of PPAR γ (Figure 6B). The inhibition of PPAR γ by berberine in RAW264.7 cells could also be confirmed by the suppression of other PPAR γ target genes, such as adipose fatty

acid-binding protein (aP2) and lipoprotein lipase (LPL) (Figure 6C). The expression of these two genes was greatly inhibited in berberine-treated RAW264.7 cells (Figure 6C). Consistently, berberine increased PPAR γ phosphorylation in these cells (Figure 6D).

Discussion

By screening herb compounds for induction of LDL receptor expression in HepG2 cells, berberine was identi-

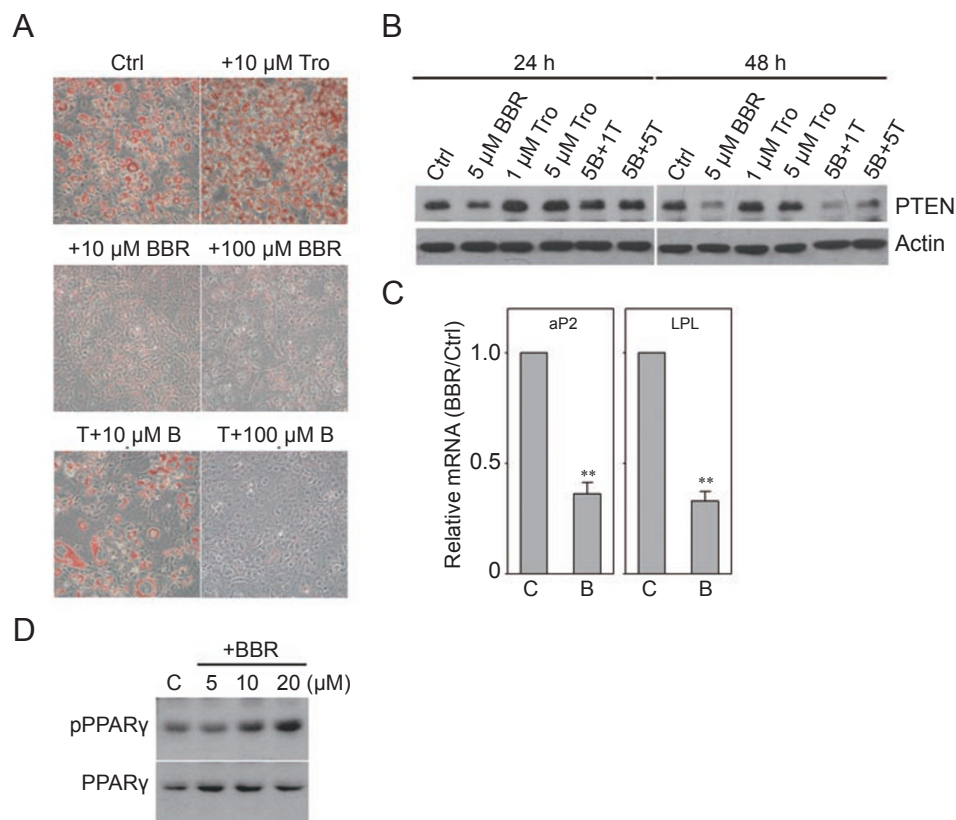


Figure 6 Berberine inhibits PPAR γ activity in RAW264.7 cell. **(A)** 3T3-L1 adipocyte differentiation inhibited by berberine. 3T3-L1 adipocyte differentiation was induced with the standard protocol [45, 46]. Berberine (+10 μ M BBR and +100 μ M BBR), troglitazone (+10 μ M Tro) or both (T+10 μ M B and T+100 μ M B) was added from day 0. Oil-Red-O staining was carried out on day 10. **(B)** Berberine inhibits troglitazone-induced PTEN expression in RAW264.7 cell. RAW264.7 cells were treated with berberine, troglitazone or both for the indicated times. PTEN was detected by western blot. **(C)** Real-time RT-PCR analysis for PPAR γ target genes in berberine-treated RAW264.7 cell. The mRNA of target genes in RAW264.7 cells treated with or without (C) 10 μ M berberine for 48 h (B) was determined by real-time RT-PCR. aP2, adipose fatty acid-binding protein; LPL, lipoprotein lipase. The results represent three independent experiments. ** $P < 0.01$. **(D)** Berberine induces PPAR γ phosphorylation in RAW264.7 cell. Cells were treated with berberine for 2 h at indicated concentration. Phosphorylated PPAR γ (pPPAR γ) and PPAR γ protein (PPAR γ) were detected by western blot.

fied to stabilize LDL receptor mRNA [5, 6]. Subsequently, it was found to reduce serum cholesterol in human and hamster [5]. However, whether berberine treatment reduces the risk of atherosclerosis in human, as other cholesterol-reducing drugs do, requires further investigation. As a model for human atherosclerosis, apoE^{-/-} mice were used to evaluate the effect of berberine on atherosclerosis development. In this mouse model, berberine aggravated the development of atherosclerosis by promoting *in vivo* foam cell formation (Figure 1). This was confirmed by *in vitro* studies in mouse and human primary macrophages, as well as in mouse RAW264.7 cells (Figure 2). Thus, the induction of foam cells by berberine may counter-balance its beneficial effects of cholesterol reduction. For example, lovastatin, a statin drug for lowering serum cholesterol and reducing the risk of

atherosclerosis, does not promote macrophage foam cell formation (Supplementary information, Figure S3). Our results suggest that to evaluate the effect of a cholesterol-reducing compound on atherosclerosis, its effect on the cells involved in atherosclerosis development, such as macrophages, should also be considered.

Atherosclerosis is a chronic inflammation condition resulting from the interaction between lipoproteins, monocyte-derived macrophages, T lymphocytes and other cellular elements in the arterial wall [9-11]. Many cytokines, such as interleukins and macrophage-associated cytokines, are locally expressed in the atherosclerotic lesions and critically involved in the pathogenesis of this disease. They are mediators of the immuno-inflammatory response and could coordinate the cell interactions [11, 35]. Berberine is also reported to have anti-inflammatory

effects *in vitro* and *in vivo* [36-38]. The administration of neutral sulfate berberine significantly reduced plasma tumor necrosis factor- α , interferon- γ and nitric oxide levels in mice challenged with lipopolysaccharide [38]. It is well known that anti-inflammation favors the protection from atherosclerosis. The anti-inflammation effect of berberine could have beneficial impact on preventing atherosclerosis. However, our study showed that berberine promoted atherosclerosis development in apoE^{-/-} mice. It is possible that the induction of foam cells by berberine overrides its beneficial effects of anti-inflammation in this mouse model for atherosclerosis.

The cholesterol-reducing effect of berberine in human and hamster results from the increased LDL receptor level in the liver [5]. However, in apoE^{-/-} mouse, berberine treatment did not increase the LDL receptor mRNA or protein in the liver (Supplementary information, Figure S4). These results may explain why berberine does not alter the serum cholesterol and lipids in apoE^{-/-} mouse (Figure 1B). Despite the difference in lowering serum cholesterol between mouse and human, the effect of berberine on promoting foam cell formation is similar in both mouse and human macrophages (Figure 2 and Figure 4). Because the serum cholesterol level is not altered in berberine-treated apoE^{-/-} mouse, the aggravation of atherosclerosis appears to be related to the effect of berberine on SR-A expression in macrophages (Figure 4). As berberine targets multiple types of cells, such as hepatocytes, adipocytes, muscle cells and macrophages (Figure 2 and Figure 4) [5-8], the animal study in apoE^{-/-} mouse is essential for understanding its potential effect in human atherosclerosis.

The mechanism of berberine-induced foam cell formation is examined using RAW264.7 cells and primary macrophages (Figure 3-5). SR-A increase appears to be the major effect through which berberine alters the cholesterol homeostasis in macrophages (Figure 3 and Supplementary information, Figure S5). In our current study, berberine induces SR-A expression through the activation of PI3-kinase signaling cascade (Figure 5 and Supplementary information, Figure S2). The involvement of PI3-kinase signaling cascade in SR-A expression is also found in human vascular smooth muscle cells [39]. The temporal order of berberine's effects on PI3-kinase signaling cascade and PPAR γ supports the fact that PPAR γ is the target of berberine (Figure 5 and Figure 6) [7, 8]. Berberine-induced PPAR γ phosphorylation precedes the decrease of PTEN and the activation of Akt (Figure 5C, 5D, 6D and Supplementary information, Figure S2). Only after prolonged berberine treatment are berberine's effect on PTEN and Akt observed. As in adipocytes and myotubes [7, 8], PPAR γ might also be

the target of berberine in macrophages (Figure 6). The detailed mechanism of berberine-induced PPAR γ phosphorylation is under investigation.

Materials and Methods

Materials

Anti-SR-A antibody was from Serotec. Anti-CD36 antibody was from Cascade Bioscience. Anti-p85 α , anti-p110 α , anti-PPAR γ , anti-Akt-1 and anti-phospho-Akt (Ser⁴⁷³-phosphorylated form) antibodies were from Santa Cruz Biotechnology. Anti-phospho-PPAR γ antibody was from Upstate Biotechnology. Anti-PTEN, horseradish peroxidase-conjugated and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies and berberine were from Sigma. Lipoprotein-deficient FBS and 3, 3'-dioctadecylcyanoacrylate perchlorate-labeled acetylated LDL (DiO-Ac-LDL) were from Biomedical Technologies. LY294002 and dipalmitoyl-L-alpha-phosphatidylinositol-3, 4, 5-trisphosphate were from Calbiochem.

Animal experiments

ApoE^{+/+} mice (C57BL/6J) from the Jackson Laboratory (Bar Harbor, ME, USA) were housed at 22 \pm 2 $^{\circ}$ C, 55% \pm 5% relative humidity, with a 12-h light/dark cycle. 8-week-old male mice (n = 10 per group) were placed on a Western diet (21% fat, 0.21% cholesterol; D12079B; Research Diets Inc.) and received daily intraperitoneal injection of either vehicle or berberine (5 mg/kg/day) for 15 weeks. After an overnight fasting period, 100 μ l blood was drawn by tail bleeding. The serum lipids were determined by enzymatic procedures (Wako Chemicals).

For histological and immunohistochemical analyses, mice were sacrificed and hearts were perfused with 20 ml saline and 10 ml 3.7% buffered formalin. Serial cryosections of 10- μ m thickness were taken from the region of the proximal aorta through the aortic sinuses and stained with Oil-red-O. The lesion size was quantified by measuring Oil-red-O stained area. The mean lesion size for each individual mouse was calculated from four sections, starting at the appearance of the tricuspid valves [40]. SR-A in cells at atherosclerotic lesion was stained with anti-SR-A antibody. 3, 3'-diaminobenzidine (Sigma) was used to reveal SR-A by brown colorization. Cell nuclei were counterstained with hematoxylin.

For western blot of SR-A in aorta sample, apoE^{+/+} mice (n = 6 per group) fed on Western diet and treated with berberine (5 mg/kg/day) for 4 weeks were sacrificed and perfused with 20 ml saline; the aorta were immediately excised and frozen in liquid nitrogen. After tissue homogenization, protein extract was subjected to western blot for SR-A.

All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Biological Research Ethics Committee of Shanghai Institutes for Biological Sciences.

In vivo and *in vitro* foam cell formation

8-week-old male apoE^{+/+} mice (n = 6 per group) were treated by berberine (2.5 mg/kg/day or 5 mg/kg/day) or vehicle control for 2 weeks and the peritoneal macrophages were collected as described by Takahashi *et al.* [41]. 3 days after peritoneal injection of 1 ml 3% thioglycolate broth, macrophages were obtained by peritoneal

lavage with Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Cells were plated at 1.0×10^6 per ml of DMEM with 10% FBS. After incubation for 3 h at 37 °C, the non-adherent cells were washed away and the adherent cells were collected for western blot, cholesterol measurement or Oil-red-O staining.

Mouse peritoneal macrophages collected from 8-week-old male C57BL/6 mice were cultured in DMEM with 10% FBS for 24 h and treated with berberine for additional 48 h. Mouse RAW264.7 cells were plated (5×10^5 cells per 35-mm dish), cultured in DMEM with 10% FBS for 48 h and treated with berberine for additional 48 h. The cells were harvested or fixed for Oil-red-O staining.

Human primary monocytes were isolated from the blood of healthy normal lipidermic volunteers of age between 25 and 30 years with a life-time non-smoking history, no acute or chronic illness, and no current medication [42, 43]. Peripheral blood mononuclear cells isolated by Ficoll-Hypaque gradient centrifugation were suspended in DMEM with 10% heat-inactivated FBS and seeded into 60-mm dishes (8×10^5 cells/ml). After 2 h incubation, the non-adherent cells were washed away and the medium was replaced with macrophage-SFM medium (Gibco) supplemented with 500 U/ml of granulocyte-macrophage colony-stimulating factor (PetroTech) and penicillin (100 U/ml)-streptomycin (100 µg/ml). After 7 days, the adherent cells were used as human monocyte-derived macrophages. On day 7, cells were treated with berberine in DMEM with 10% FBS for 24 h and then harvested or fixed for Oil-red-O staining.

Cholesterol measurement

Cells were washed with phosphate-buffered saline (PBS). The cellular lipids were obtained by sonicating the cells in hexane/isopropanol (3/2, v/v) and extracting for 24 h. After removing cell debris by centrifuging at 12 000 g, the supernatant was dried under nitrogen flush and re-dissolved in isopropanol. Total cholesterol was determined with an Amplex Red Cholesterol Assay Kit (Invitrogen) [44]. The protein was determined by Lowry assay.

Western blot

Cells were washed with ice-cold PBS, lysed directly in boiling 1× Laemmli SDS buffer containing 20 mmol/l dithiothreitol and heated at 100 °C for 5 min. Samples were subjected to western blot [45, 46].

Real-time RT-PCR

Total RNA was prepared from cells with TRIzol™ reagent (Invitrogen). Total RNA (2 µg) was reverse-transcribed with oligo-dT primer. The cDNA samples were then diluted to appropriate concentration for real-time PCR analysis (Rotor-Gene 3000, Corbett Research Pty Ltd). Hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used as a constitutive gene control. The target mRNA was normalized with HPRT in the same sample. The PCR primers are: PTEN (forward: 5'-CGG AAC TTG CAA TCC TCA GT-3'; reverse: 5'-AGG TTT CCT CTG GTC CTG GT-3'), SR-A (forward: 5'-TGG TCC ACC TGG TGC TCC-3'; reverse: 5'-ACC TCC AGG GAA GCC AAT TT-3'), aP2 (forward: 5'-AAA GAC AGC TCC TCC TCG AAG GTT-3'; reverse: 5'-TGA CCA AAT CCC CAT TTA CGC-3'), LPL (forward: 5'-GGC TCT GCC TGA GTT GTA G-3'; reverse: 5'-AGA AAT TTC GAA GGC CTG GT-3'), HPRT (forward: 5'-CCT GCT GGA TTA CAT TAA AGC-3';

reverse: 5'-TTC AAC ACT TCG AGA GGT CCT-3').

DiO-Ac-LDL Uptake

DiO-Ac-LDL uptake was performed as described by Cao *et al.* [39]. RAW264.7 cells were treated with or without berberine for 48 h, incubated in DMEM with 2% lipoprotein-deficient FBS and 10 µg/ml DiO-Ac-LDL for 4 h at 37 °C, washed with PBS and analyzed by FACScan flow cytometer (BD Biosciences). For anti-SR-A or anti-CD36 antibody-blocking experiment, cells were pre-incubated with 10 µg/ml antibody in DMEM containing 2% lipoprotein-deficient FBS for 1 h at 37 °C. DiO-Ac-LDL was then added to the cells for uptake.

Cell surface receptor measurement

RAW264.7 cells were treated with 10 µM berberine or not for 48 h, washed with ice-cold PBS containing 0.5% bovine serum albumin, and incubated with anti-SR-A, anti-CD36 or isotype blank antibody for 1 h on ice. FITC-conjugated secondary antibody was used for flow cytometry analysis.

SR-A gene promoter analysis

Human SR-A promoter fragment (−696 to +46, was kindly provided by Dr Christopher Glass, University of California, San Diego) in a luciferase reporter vector (pGL3-basic, Promega) was transfected into HEK 293T cells by Lipofectamine 2000 (Invitrogen). 24 h after transfection, cells were treated with berberine, LY294002 or both for additional 24 h. Luciferase reporter assay was carried out using the Dual-Luciferase Reporter Assay System (Promega) and the transfection efficiencies were normalized to Renilla luciferase activity.

Statistical Analysis

Data were presented as mean ± SD. Differences were analyzed by Student's *t*-test. *P* < 0.05 was considered statistically significant.

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