Cardiovascular pharmacology

Berberine-induced inhibition of adipocyte enhancer-binding protein 1 attenuates oxidized low-density lipoprotein accumulation and foam cell formation in phorbol 12-myristate 13-acetate-induced macrophages

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Abstract

The phagocytosis of oxidized low-density lipoprotein (oxLDL) by monocyte-derived macrophages and the subsequent differentiation of macrophages into foam cells are the key steps in atherogenesis. Scavenger receptors, such as CD36 and lectin-like low-density lipoprotein receptor 1 (LOX-1), are responsible for the uptake of oxLDL. Adipocyte enhancer-binding protein 1 (AEBP1) regulates many key genes associated with intracellular cholesterol efflux. The present study investigated the function of berberine, a compound isolated from Rhizoma coptidis, on foam cell formation, and explored the possible underlying mechanism. We found that berberine inhibited the oxLDL uptake of macrophages and reduced foam cell formation in a dose-dependent manner. Moreover, AEBP1 expression in macrophages increased and decreased after oxLDL and berberine treatments in a dose-dependent manner, respectively. Berberine reduced the expression of scavenger receptors CD36 and LOX-1, but did not affect the expression of CD68 in oxLDL-stimulated macrophages. Overall, berberine reduced foam cell formation by a dual mechanism, which decreased oxLDL internalization via the suppression of scavenger receptors, such as CD36 and lectin-like low-density lipoprotein receptor 1 (LOX-1), and increased cholesterol efflux by inhibiting AEBP1 expression in macrophages.

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1. Introduction

Atherosclerosis is a well-known multigenic, progressive chronic inflammatory disease. Numerous studies demonstrated that oxidized low-density lipoprotein (oxLDL) is a major risk factor of atherosclerosis (Aviram, 1993; Jalal and Devaraj, 1996). oxLDL stimulates the expression of scavenger receptors such as CD36, low-density lipoprotein receptor 1 (LOX-1), and scavenger receptor A in monocyte-derived macrophages (Mehta et al., 2006; Nakagawa et al., 1998). Moreover, oxLDL could induce macrophages into foam cells through scavenger receptors, which is the key step in the early stage of atherogenesis. The death of foam cells forms necrotic and cholesterol-rich lipid core and promotes plaque instability. Therefore, reducing the accumulation of cholesterol in macrophages might hamper foam cell formation, which indicates a possible therapeutic role in plaque stability. By contrast, increasing the efflux of intracellular cholesterol mainly through the LXRe–ABCA1 pathway is another critical mechanism to impede the initiation and progression of atherosclerotic lesions (Lee et al., 2010; Majdalawieh and Ro, 2010b). Berberine is a botanical alkaloid isolated from medicinal herbs, such as Coptidis (Huanglian) and Cortex phellodendri (Huang-bai; Ikram, 1975). Berberine has been used extensively in traditional Chinese medicine to treat infectious diarrhea (Stermitz et al., 2000) and cancer (Anis et al., 2001). Recently, increasing evidence has shown that berberine has protective effects in cardiovascular diseases. For example, berberine could decrease plasma cholesterol (Kong et al., 2004) and glucose levels (Yin et al., 2008). Moreover, berberine exerts anti-atherogenic effects by inhibiting MMP-9 and EMMPRIN expressions (Huang et al., 2011). In addition, berberine abrogates foam cell formation by suppressing lipid accumulation via the activation of JNK-activated protein kinase (Lee et al., 2007) and by enhancing LXRe–ABCA1-dependent cholesterol efflux (Lee et al., 2010). However, the role of upstream mechanism in regulating cholesterol metabolism by berberine remains unclear.

The inhibition of the PPAR-1–LXRe–ABCA1 signaling pathway inhibits atherogenesis by increasing macrophage cholesterol efflux (Majdalawieh et al., 2006). In addition, recent evidence has suggested that adipocyte enhancer-binding protein 1 (AEBP1)
is a transcriptional repressor that impedes macrophage cholesterol efflux, thereby promoting foam cell formation (involved in macrophage cholesterol homeostasis) by downregulating PPAR-1 and LXRe expressions (Majdalawieh et al., 2006). Accordingly, we speculate that berberine might have an effect on macrophages by regulating AEBP1 expression. The present study investigated the effect of berberine on cholesterol homeostasis in phorbol 12-myristate 13-acetate (PMA)-induced macrophages and clarified berberine's effect on AEBP1 expression in oxLDL-stimulated macrophages.

2. Materials and methods

2.1. Reagents and instruments

RPMI 1640 medium, fetal bovine serum (FBS), and penicillin/streptomycin (pen/strep, 10,000 U/ml each) were purchased from Invitrogen (Carlsbad, CA, USA). PMA was obtained from Calbiochem (San Diego, CA, USA); Dimethyl sulfoxide (DMSO), berberine, and oil red were acquired from Sigma-Aldrich (St. Louis, MO, USA). OxLDL was purchased from Beijing Union Medical Biochemistry Room (Beijing, China), whereas Dil-labeled oxLDL (dil-oxLDL) was purchased from the Guangzhou Zhongshan University School of Public Health (Guangzhou, China). FITC (Fluorescein isothiocyanate)-conjugated anti-CD36 (NL07) and R-phycocerythrin (PE)-conjugated anti-CD68 antibody (Y1/82A) were obtained from eBioscience (San Diego, CA, USA). FITC-conjugated anti-LOX-1 antibody (23C11) was acquired from Hycult Biotechnology (Uden, Netherlands). The mouse monoclonal antibody to AEBP1 (ab54820) was produced by Abcam (Cambridge, UK). Waters 2695 Alliance System (Waters Corporation, Milford, MA, USA) was used for high-performance liquid chromatography (HPLC) analysis. Cholesterol standards were obtained from China National Pharmaceutical Group Chemical Reagent Company (Beijing, China). Isopropyl alcohol, heptane, acetonitrile, n-hexane, potassium hydroxide, ethanol, and trichloroacetic acid were purchased from China Medicine Group Shanghai Chemical Reagent Company (Shanghai, China).

2.2. Cell culture and treatment

Human monocytic cell line THP-1 was purchased from American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI 1640 medium containing 10% FBS, 10 mM HEPES (Sigma), and 1% pen/strep solution at a density of 5 × 10^5 cells/ml in a 5% CO₂ incubator. The cells were seeded in six-well plates for 48 h in the presence of 100 nM PMA, which allowed them to differentiate into adherent macrophages (Huang et al., 2011). The culture medium was then changed into RPMI 1640 medium containing 0.5% FBS for 6 h of cell starvation. To observe AEBP1 expression, the cells were stimulated with oxLDL (25 μg/ml–50 μg/ml) for 24 h. The macrophages were pretreated with berberine (0–50 μM) for 1 h, stimulated with oxLDL (50 μg/ml) or dil-oxLDL (50 μg/ml) for 24 h, and then collected for detection.

2.3. Oil red staining

After the above mentioned treatment, the cells were collected, washed twice with ice-cold phosphate-buffered saline (PBS), and then fixed with 10% neutral-buffered formalin for 15 min. The cells were washed again with 60% isopropl alcohol for 1 min and then stained with oil red for 15 min. The oil red was discarded, and excess dye was washed away with 60% isopropyl alcohol. After washing with distilled water, the specimens of each group were placed under an inverted microscope to observe oil red staining.

2.4. Flow cytometry

Flow cytometry was performed using primary mouse anti-human monoclonal antibodies FITC–anti-CD36, PE–anti-CD68, and FITC–anti-LOX-1 according to the manufacturer's protocol. The cells were analyzed on a BD FACS Calibur. To observe the effect of berberine on the intake of oxLDL, the cells were treated with Dil-labeled oxLDL instead of oxLDL. The mean fluorescence intensity of dil oxLDL in macrophages was analyzed on a BD FACS Calibur.

2.5. Lipid extraction from cells and HPLC analysis

After the abovementioned treatment, the cells were collected in sterile PBS, lysed via ultrasonic frozen pyrolysis (power 600 W, working 4 s, interval 8 s, and repeated 20 times), and then centrifuged at 12879g for 10 min at 4 °C to remove cell debris. Viable cells were then divided into two aliquots. One aliquot was added with an equal volume of freshly prepared 15% potassium hydroxide (KOH) alcohol solution and vortexed at room temperature to obtain total cholesterol from cell lysate. The other aliquot was added with an equal volume of freshly prepared 8.9 mmol/L of KOH alcohol solution and then placed in a water bath at 80 °C for 1 h to obtain free cholesterol. To remove the protein, 6% trichloroacetic acid was added. Subsequently, an equal volume of hexane/isopropanol solution (4:1, v/v) was added. The mixture was vortexed for 5 min and then centrifuged at 201g for 5 min. Total lipid extracts were dried in an evaporator and redissolved in isopropanol:heptane:acetonitrile (35:12:52, v/v/v) for HPLC analysis. Separation of free cholesterol and cholesteryl esters was conducted as previously described (Cullen et al., 1997) with an Agilent 1100 HPLC system (Agilent, Palo Alto, CA). A C18 column with isopropyl alcohol:heptane:acetonitrile as the mobile phase was used for non-gradient elution at a flow rate of 1 ml/min and a column temperature of 4 °C. Detection was performed at 216 nm for 12 min. The peak area quantified cholesterol. Cholesterol esters were hydrolyzed using cholesterol esterase, and total cholesterol was measured. The amount of cholesterol esters could be calculated by deducing free cholesterol from total cholesterol. Each sample in the assays was done in triplicate, and at least three independent experiments were performed.

2.6. Protein isolation and Western blot analysis

Protein isolation and Western blot analysis of cell lysate were performed as previously described (Huang et al., 2008), except that the membranes were probed with the mouse primary AEBP-1 antibody (diluted 1:1000 in TBST) for 2 h or rabbit anti-actin (diluted 1:5000 in TBST) for 1 h, and then incubated with goat anti-rabbit secondary antibody labeled with far-red-fluorescent Alexa Fluor 680 dye. Briefly, all signals were detected on an Odyssey imaging system (Li-cor, USA). Densitometric analysis was performed on a Quantity One system (Bio-Rad) to scan the signals.

2.7. Statistics

All experiments were repeated three times and presented as mean ± S.D. Comparisons among three groups or more were analyzed via one-way ANOVA and post-hoc analysis using the least significance difference, Student–Newman–Keuls, and Dunnet methods. All data were analyzed with SPSS 11.5 software.
3. Results

3.1. Effects of berberine on oxLDL accumulation in PMA-induced macrophages

Macrophages were pretreated with berberine for 1 h and then stimulated with oxLDL (50 μg/ml) for 24 h. Intracellular lipid droplets were observed through oil red staining. As shown in Fig. 1(A)–(E), oxLDL-stimulated macrophages showed much more red-stained lipid droplets than the control group without oxLDL. However, this trend was inhibited by berberine pretreatment. Furthermore, oxLDL was replaced with Dil-oxLDL to treat cells, and then the Dil fluorescence intensity was detected through flow cytometry. Consistent with the oil red staining results, berberine significantly inhibited the phagocytosis of oxLDL in macrophages (Fig. 1F).

3.2. Effects of berberine on ratio of cholesteryl esters to total cholesterol in macrophages

Foam cell formation is due to cholesteryl esters accumulation and occurs when the cholesteryl esters content accounts for more than 50% of total cholesterol (Maor and Aviram, 1994). Therefore, we further traced intracellular cholesteryl esters via HPLC to determine the effects of berberine on the formation of foam cells. The data (Fig. 2A) showed that oxLDL treatment increased cholesteryl esters/total cholesterol ratio compared with the control group without oxLDL treatment (68.03 ± 1.76% vs. 34.46 ± 1.29%, P < 0.01). However, berberine pretreatment inhibited oxLDL-induced foam cell formation in a dose-dependent manner (5 μM–50 μM from 62.37 ± 2.51% to 44.4 ± 3.08%, respectively).

3.3. Effects of berberine on expression of scavenger receptors CD36, LOX-1, and CD68 at the surface of macrophages

Macrophages phagocytose oxLDL through scavenger receptors, including CD36, LOX-1, CD68, and scavenger receptor A, to form foam cells. To elucidate the molecular mechanism of berberine inhibition on the accumulation of oxLDL in macrophages, we detected the expression of scavenger receptors. As depicted in Fig. 2B, berberine suppressed CD36 and LOX-1 expressions in oxLDL-simulated macrophages in a dose-dependent manner. Furthermore, the inhibition rate of CD36 and LOX-1 expressions varied from −7.5% to −21.3% (P < 0.01) and −7.3% to −25.3% (P < 0.01), respectively, but showed no effect on CD68 expression.

3.4. Effect of oxLDL and berberine on AEBP1 expression in macrophages

One emerging concept showed that AEBP1 is a critical regulator of macrophage cholesterol homeostasis and foam cell formation. To investigate further the possible underlying mechanism and determine whether berberine reduced oxLDL phagocytosis involved in the cholesterol efflux from macrophages, the cells were treated with oxLDL (25 μg/ml–50 μg/ml) for 24 h and

Fig. 1. Effects of berberine on the accumulation of oxLDL in PMA-induced macrophages. Cells were pretreated with various concentrations of berberine (10, 25, and 50 μM) or vehicles for 1 h and then cultured with oxLDL or Dil-oxLDL (50 μg/ml) for 24 h. The amount of intracellular oxLDL was assessed via oil red staining and flow cytometry. (A)–(E) Representative images of oil red staining. (F) Results of average fluorescence intensity of Dil-oxLDL in macrophages. Data (mean ± S.D.) were obtained from three independent experiments. ***: P < 0.01 compared with the control, *: P < 0.01 compared with ** marked group.
collected for immunoblot analysis. The results (Fig. 3) showed that AEBP1 expression greatly increased in oxLDL-induced macrophages. Furthermore, the increasing AEBP1 expression was hampered by berberine pretreatment in a dose-dependent manner.

4. Discussion

Lipid accumulation, especially oxLDL, in macrophages promotes foam cell formation (Lusis, 2000; Ross, 1999), which enlarges the lipid core and increases plaque vulnerability. Therefore, inhibiting the excessive intake of oxLDL and reducing foam cell formation have important theoretical and practical significance on the stability of atherosclerotic plaque. In the present study, berberine significantly reduced the accumulation of oxLDL in PMA-induced macrophages. This suppressive effect of berberine on foam cell formation is consistent with previous data showing that berberine treatment reduces serum cholesterol and impedes the development of atherosclerosis (Kong et al., 2004). The results of the present study suggested that the effect of berberine-mediated alleviation on foam cell formation in PMA-induced macrophages might be partly attributed to the inhibition of CD36 and LOX-1 expressions. By contrast, Li et al. (2009) reported that berberine alone significantly increases lipid...
acumulation in murine macrophages (Raw264.7) without oxLDL stimulation and exacerbates the progression of atherosclerosis in apolipoprotein E-deficient mice. The discrepancy in results could be due to differences in cell types and experimental conditions.

HPLC analysis showed that the cholesterol esters/total cholesterol ratio of oxLDL-treated macrophages was above 50% compared with the blank control group. This result indicates that the formation of foam cell took place. Intracellular cholesterol esters/total cholesterol ratio decreased in a dose-dependent manner after berberine pretreatment. Experimental data revealed that the inhibitory effect of berberine reached its maximum at the concentration of 50 μM, and the cholesterol esters/total cholesterol ratio decreased below 50%. These results strongly suggest that berberine inhibits macrophages from uptaking oxLDL, which reduces the degree of foam cell formation. We hypothesized that berberine may play an anti-atherogenesis role to a certain extent (at least in vitro).

The intracellular cholesterol homeostasis in macrophages is regulated dynamically by cholesterol intake and efflux via a different mechanism. Among these, the PPAR-α-LXRα–ABCA1 (ATP binding box transporter A1) pathway plays a major role in cholesterol efflux (Majdalawieh and Ro, 2010b), and berberine increases cholesterol efflux in macrophages by interfering with the LXRs–ABCA1 pathway (Lee et al., 2010). As a result, cholesterol accumulation in macrophages is reduced and foam cell formation is prevented. Moreover, AEBP1 can regulate PPAR-α and LXRα expression levels directly (Majdalawieh et al., 2006). Accordingly, we further investigated AEBP1 expression in oxLDL-stimulated macrophages. In addition, the effect of berberine on AEBP1 expression was studied. AEBP1 is a 82 kDa intercellular protein impeding cholesterol clearance from macrophages by downregulating PPAR-α and LXRs expression (Majdalawieh and Ro, 2010a; Majdalawieh et al., 2006). The ablation and overexpression of AEBP1 increased and decreased the levels of ABCA1, ABCG1, and ApoE in macrophages. AEBP1-knockout (AEBP1−/−) macrophages exhibit enhanced cholesterol efflux compared with wild-type (AEBP1+/+) macrophages (Majdalawieh et al., 2006). Therefore, the positive regulation of foam cell formation by AEBP1 is consistent with its ability to repress PPAR-α and LXRs transcriptionally in macrophages. In addition, AEBP1 developed its pro-inflammatory responsiveness by increasing IL-6, MCP-1, and TNF-α expressions. These correlations strongly indicate that AEBP1 manifests itself as a potential pro-atherogenic factor and serves as a potential molecular target for impeding foam cell formation, inhibiting inflammation, and subsequently suppressing atherogenesis. In the current study, AEBP1 expression in macrophages was increased and suppressed by oxLDL and berberine in a dose-dependent manner, respectively. Thus, berberine regulates foam cell formation not only by decreasing the internalization of oxLDL via suppression of CD36 and LOX-1 but also by increasing cholesterol efflux through inhibition of AEBP1 expression.

Interestingly, AEBP1 upregulates the expression of NF-κB (Majdalawieh and Ro, 2010a; Majdalawieh et al., 2006) and represses PPAR-α, LXR and ABCA1 (Majdalawieh and Ro, 2010; Majdalawieh et al., 2006). These events significantly promote cholesterol efflux in macrophages by inhibiting NF-κB activation (Ferreira et al., 2007; Lee et al., 2010). AEBP1 possibly regulates foam cell formation through NF-κB activation followed by PPARs–LXRs–ABCA1 suppression. Therefore, the inhibition of AEBP1 expression might benefit intracellular cholesterol metabolism. As expected, berberine was found to significantly reduce AEBP1 expression and then foam cell formation. Furthermore, previous data indicated that berberine inhibits NF-κB activation in oxLDL-induced macrophages (Huang et al., 2012) and downregulates the LXRs–ABCA1 pathway (Lee et al., 2010). Therefore, we speculate that AEBP1 serves as an upstream mechanism that regulates the amount of intracellular cholesterol in macrophages, which satisfactorily explains berberine’s properties in oxLDL-induced macrophages.

In addition, the pharmacokinetic property of oral absorption of 1.2 g of berberine has been reported for healthy volunteers and patients with ischemic heart failure. The T (peak) was 2.37 ± 0.04 h, and the Cmax was 394.7 ± 154.4 ng/ml (Zeng, 1998). In this study, we showed that 5–50 μM berberine attenuated oxLDL accumulation and foam cell formation in macrophages, and the experimental dose of berberine (50 μM) is equivalent to a daily oral administration of 0.3 g berberine. Collectively, berberine would be a promising candidate as a therapeutic agent applied to a relative in vivo study.

In conclusion, berberine pretreatment reduces macrophage phagocytosis of oxLDL and cholesterol efflux and thus inhibits foam cell formation. Berberine inhibits the expression of scavenger receptors CD36 and LOX-1 as well as the activation of the AEBP1–NF-κB–PPARs–LXRs–ABCA1 pathway to increase intracellular reverse output of cholesterol and thereby reduce foam cell formation.

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References


Majdalawieh, A., Ro, H.S., 2010a. Regulation of Ikappa-Balpha function and IkappaBalpha-PPARs–LXRs–ABCA1 suppression. Therefore, the inhibition of AEBP1 expression and then foam cell formation. Furthermore, previous data indicated that berberine inhibits NF-κB activation in oxLDL-induced macrophages (Huang et al., 2012) and downregulates the LXRs–ABCA1 pathway (Lee et al., 2010). Therefore, we speculate


