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Anti-Atherogenic Effect of Berberine on LXRα-ABCA1-Dependent Cholesterol Efflux in Macrophages

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ABSTRACT

Berberine, a botanical alkaloid purified from *Cortidis rhizoma*, has effects in cardiovascular diseases, yet the mechanism is not fully understood. Foam cells play a critical role in the progression of atherosclerosis. This study aimed to investigate the effect of berberine on the formation of foam cells by macrophages and the underlying mechanism. Treatment with berberine markedly suppressed oxidized low-density lipoprotein (oxLDL)-mediated lipid accumulation, which was due to an increase in cholesterol efflux. Berberine enhanced the mRNA and protein expression of ATP-binding membrane cassette transport protein A1 (ABCA1) but did not alter the protein level of ABCG1 or other scavenger receptors. Additionally, functional inhibition of ABCA1 with a pharmacological inhibitor or neutralizing antibody abrogated the effects of berberine on cholesterol efflux and lipid accumulation. Moreover, berberine induced the nuclear translocation and activation of liver X receptor α (LXRα) but not its protein expression. Knockdown of LXRα mRNA expression by small interfering RNA abolished the berberine-mediated protective effects on ABCA1 protein expression and oxLDL-induced lipid accumulation in macrophages. These data suggest that berberine abrogates the formation of foam cells by macrophages by enhancing LXRα-ABCA1-dependent cholesterol efflux. J. Cell. Biochem. 111: 104–110, 2010.

KEY WORDS: BERBERINE; LXRα; ABCA1; CHOLESTEROL EFFLUX; MACROPHAGE FOAM CELL

Regulation of cholesterol metabolism by macrophages is critical in the initiation and progression of atherosclerotic lesions. Increased level of modified low-density lipoprotein (LDL) promotes inflammation to recruit monocytes to the intima, where monocytes differentiate into macrophages to engulf lipoproteins. The internalized lipoproteins are processed, stored and progressively accumulated in cytoplasmic droplets, thus leading to the formation of foam cells and the progression of early to intermediate atherosclerotic lesions [Li and Glass, 2002; Rader and Pure, 2005]. Several types of macrophage scavenger receptors (SRs), including SR-A and CD36, are responsible for the internalization of oxidized LDL (oxLDL) and promote cholesterol accumulation in macrophages [de Winther and Hofker, 2000; Febbraio et al., 2000; Kunjathoor et al., 2002]. In contrast, the efflux of intracellular cholesterol is mediated by reverse cholesterol transporters (RCTs), including SR-BI, ATP-binding membrane cassette transport protein A1 (ABCA1) and G1 (ABCG1) [Ohashi et al., 2005; Cuchel and Rader, 2006; Wang and Rader, 2007].

Berberine, a botanical alkaloid isolated from the Chinese herb huanglian (*Coptis rhizoma*), has been used in Chinese medicine to treat infectious diarrhea, dysentery, stomatitis, throat infections, and hepatitis [Lau et al., 2001; Cheng et al., 2009]. Recently, increasing studies have suggested that berberine has protective effects in cardiovascular diseases. For example, berberine reduces the serum level of cholesterol by upregulating hepatic LDL receptor expression and suppresses lipid accumulation via activation of

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AMP-activated protein kinase [Kong et al., 2004; Lee et al., 2007]. Berberine also exerts anti-atherogenic effects by promoting the activation of endothelial nitric oxide synthase in endothelial cells and inhibiting the proliferation of vascular smooth muscle cells and the formation of neointima in balloon injury models [Lee et al., 2006; Holy et al., 2009]. Moreover, berberine has anti-diabetic activities by preventing hyperglycemia-induced endothelial injury and adipocyte differentiation and by increasing insulin secretion and sensitization [Leng et al., 2004; Brusq et al., 2006; Huang et al., 2006; Kong et al., 2009; Wang et al., 2009]. These results suggest that berberine has multiple protective roles in cardiovascular and related physiological functions. However, the effect of berberine on macrophage foam cell formation and intracellular cholesterol metabolism remains unexplored.

In the present study, we aimed to investigate the molecular mechanism underlying the anti-atherogenic effect of berberine on macrophages. We first investigated the effect of berberine on cholesterol homeostasis in human THP-1-derived macrophages, then delineated its effect on the expression of SR-A, CD36, SR-BI, ABCA1, and ABCG1. Finally, we explored the underlying molecular mechanisms by which berberine induces changes in the expression of these SRs and RCTs and participates in foam cell formation. Berberine increased cholesterol efflux through a liver X receptor α (LXRα)-ABCA1 pathway, which resulted in reduced cholesterol accumulation in macrophages to prevent the formation of foam cells.

MATERIALS AND METHODS

REAGENTS

Berberine, phorbol myristate-acetate (PMA), Oil-red O, LDL and diisothiocyanatostilbene-2, 20-disulphonic acid disodium salt (DIDS) were from Sigma–Aldrich (St. Louis, MO). Protease inhibitor cocktail was from Roche (Basel, Switzerland). Antibodies against ABCA1, ABCG1, SR-BI, and SR-A were from Novus Biologicals (Littleon, CO). Antibodies against LXRα, RXR, CD36, lamin A/C, α-actin, α-tubulin, control siRNA and LXRα siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG and rhodamine red-conjugated donkey anti-rabbit IgG antibodies and HRP-conjugated goat anti-rabbit IgG and anti-mouse IgG antibodies were from Jackson Immuno Research (West Grove, PA). 3-Dodecanoyl-NBD-cholesterol (NBD-cholesterol) was from Cayman (Ann Arbor, MS). Cholesterol and triglyceride assay kits were from RANDOX (Antrim, UK).

CELL CULTURE

THP-1 cells, a human acute monocytic leukemia cell line, were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin and 50 units/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were treated with PMA (50 ng/ml) for 72 h at 37°C to differentiate into adherent macrophages.

OXIDATION OF LDL

Oxidation of LDL was as described [Lin et al., 2007]. Briefly, native LDL was dialyzed against phosphate-buffered saline (PBS) containing 10 μM $CuSO_4$ at 37°C for 24 h. The oxidation of LDL was indicated by the characteristic color change from golden to yellow to translucent without color. The oxidation was stopped by dialyzing against PBS containing 0.3 mM EDTA at 4°C overnight. The extent of oxidation was monitored by measuring thiobarbituric acid-reactive substances.

OIL-RED O STAINING FOR CYTOPLASMIC LIPID DETECTION

Oil-red O staining was as described [Ramirez-Zacarias et al., 1992]. Cells were fixed with 4% paraformaldehyde and then stained with 0.5% Oil-red O. Hematoxylin was used for counterstaining. The density of lipid content was evaluated by alcohol extraction after staining. The absorbance at 540 nm was measured on a microplate reader (BioTek Instruments, Winooski, VT).

CHOLESTEROL MEASUREMENT

Cellular cholesterol was extracted by use of hexane/isopropanol (3/2, v/v). After removing cellular debris, the supernatant was dried under nitrogen flush. The level of cholesterol was measured by use of a cholesterol assay kit.

CHOLESTEROL EFFLUX ASSAY

Macrophages were treated with various concentrations of berberine (5, 10, and 20 μM) for 12 h, then equilibrated with NBD-cholesterol (1 μg/ml) for an additional 6 h in the presence of berberine. NBD-cholesterol–labeled cells were washed with PBS and incubated in RPMI 1640 medium for 6 h. The fluorescence-labeled cholesterol released from the cells into the medium was measured by use of a multilabel counter (PerkinElmer, Waltham, MA). Cholesterol efflux was expressed as percentage fluorescence in the medium relative to total fluorescence.

WESTERN BLOT ANALYSIS

Cells were lysed with RIPA buffer (98% PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail). Nuclear extracts of macrophages were prepared as described [Chang et al., 2005]. Aliquots (50 μg) of cell lysates or 20 μg nuclear extracts were resolved on SDS-PAGE and examined by Western blot analysis as described [Shyue et al., 2001].

RT-PCR

Total RNA was extracted from cells with use of TRI regent (Sigma). In total, 1.5 μg RNA was used for cDNA synthesis with oligo (dT) and SuperScript III reverse transcriptase (Invitrogen). cDNAs were used for PCR amplification with the primers 5′-AAC AGT TTG TGG CCC TTT TG-3′ and 5′-AGT TCC AGG CTG GGG TAC TT-3′ for *ABCA1* (157 bp). PCR of α-actin was used as a reference with the primers 5′-GGG TCA GAA GGA TTC CTA TG-3′ and 5′-GGT CTC AAA CAT GAT CTG GG-3′ (238 bp). The PCR amplification was 25 cycles of 95°C for 30 s, 58°C for 40 s, and 72 °C for 40 s.

IMMUNOFLUORESCENCE ASSAY

THP-1-derived macrophages on coverslips were treated with berberine for 6 h. Macrophages fixed in 4% paraformaldehyde were permeablized with methanol for 15 min. Cells were blocked with 10% FBS and then incubated with rabbit anti-LXRα (1:50)

antibody for 1 h. After being washed 3 times, cells were incubated with rhodamine red-labeled donkey anti-rabbit IgG for 1 h. The cells were counterstained with DAPI. After a washing, cells were mounted and analyzed on confocal microscopy.

TRANSIENT TRANSFECTION AND LUCIFERASE REPORTER ASSAY

For promoter activation assay, cells were transfected with 3xLXRE-Luc plasmid, a reporter construct containing three copies of LXRE, by use of lipofectamine. pGL3-renilla was co-transfected as a control. Twenty-four hours after transfection, cells were treated with berberine for an additional 24 h. The cells were then lysed for luciferase and renilla activity assays (Promega).

STATISTICAL ANALYSIS

All data represent the mean ± SEM from at least three independent experiments. Statistical analyses involved one-way ANOVA with post hoc Fisher LSD test. Statistical significance was inferred at $P < 0.05$.

RESULTS

BERBERINE ATTENUATES OXLDL-INDUCED CHOLESTEROL ACCUMULATION AND INCREASES CHOLESTEROL EFFLUX IN MACROPHAGES

To investigate the effect of berberine on oxLDL-induced foam cell formation, THP-1-derived macrophages were treated with oxLDL for 24 h, which led to lipid accumulation, as revealed by measurement of intracellular lipid or cholesterol content. The increase in lipid accumulation was markedly attenuated by treatment with berberine in a dose-dependent manner (Fig. 1A–C). Moreover, cholesterol efflux was evaluated by use of fluorescent NBD-cholesterol. Berberine dose-dependently increased the efficiency of cholesterol efflux in macrophages (Fig. 1D), which suggests that

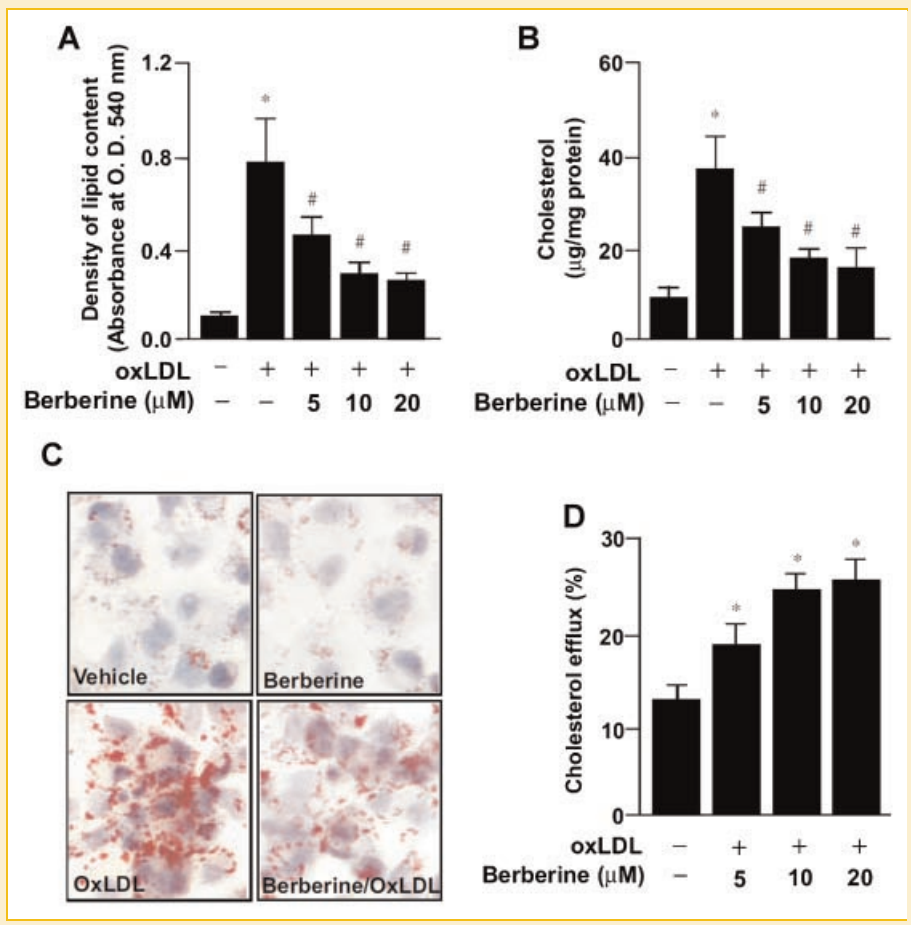

Fig. 1. Berberine attenuates lipid accumulation and promotes cholesterol efflux in macrophages. THP-1-derived macrophages were co-treated with the indicated concentrations of berberine (5, 10, and 20 μM) and oxLDL (50 μg/ml) for 24 h. Cells were fixed and stained with Oil-red O to detect intracellular lipid content. A: Density of lipid content was evaluated by alcohol extraction, and absorbance was measured at 540 nm. B: Intracellular cholesterol was extracted by use of hexane/isopropanol (3/2, v/v) and determined by an enzymatic method. C: After staining with Oil-red O, cellular nuclei were stained with hematoxylin. Magnification: 400×. D: Macrophages were treated with berberine and NBD-cholesterol as described in Materials and Methods Section. Cholesterol efflux was expressed as percentage fluorescence in the medium relative to total fluorescence. Data are mean ± SEM from three independent experiments. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus oxLDL alone. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

berberine-induced enhancement of cholesterol efflux may contribute to abrogating the formation of foam cells.

BERBERINE UPREGULATES ABCA1 EXPRESSION IN THP-1-DERIVED MACROPHAGES

To explore the possible molecular mechanisms involved in the lipid-lowering effect of berberine, we examined the effect of berberine on the expression of SRs and RCTs. Treatment with berberine for 18 h dose-dependently increased the protein level of ABCA1 but not ABCG1, SR-BI, SR-A, or CD36 (Fig. 2A–F). In addition, both the protein and mRNA levels of ABCA1 were time-dependently upregulated in macrophages (Fig. 2G,H).

INHIBITION OF ABCA1 FUNCTION ABROGATES THE LIPID-LOWERING EFFECT OF BERBERINE

To further investigate the role of ABCA1 in the lipid-lowering effect of berberine, THP-1-derived macrophages were pretreated with an ABCA1 inhibitor, DIDS [Chinetti et al., 2001], or ABCA1 neutralizing antibody in the presence of berberine. Pretreating macrophages with DIDS or ABCA1 antibody diminished the berberine-induced increase in cholesterol efflux (Fig. 3A). Additionally, pre-incubation with DIDS or ABCA1 antibody abolished the berberine-mediated suppression of oxLDL-induced lipid accumulation (Fig. 3B), which implies that induction of ABCA1 is required for berberine-mediated protection against foam cell formation.

ROLE OF LXRα ACTIVATION IN BERBERINE-MEDIATED UPREGULATION OF ABCA1 AND ABROGATION OF LIPID ACCUMULATION IN MACROPHAGES

Activation of LXRα/RXR system has been suggested to play a critical role in ABCA1 gene expression [Chawla et al., 2001]. To investigate whether LXRα is involved in berberine-induced ABCA1 upregulation, we examined the expression of LXRα and RXR in response to berberine. The nuclear level of LXRα was time-dependently increased with berberine administration (Fig. 4A), but the level of nuclear RXR (Fig. 4A) and total LXRα was not changed (data not shown). Confocal microscopy revealed LXRα distributed in both the nucleus and cytosol in control cells but translocated into the nucleus after berberine treatment (Fig. 4B). Moreover, LXRα activation assays with 3xLXRE-Luc reporter plasmid transfection revealed that berberine significantly increased LXRE-mediated luciferase activity by 1.6- to 2.7-fold in a dose-dependent manner (Fig. 4C). Transfection with various concentrations of LXRα siRNA dose-dependently decreased the protein level of LXRα in macrophages (Fig. 5A). Moreover, co-incubating berberine with LXRα siRNA abolished the berberine-induced increase in ABCA1 level (Fig. 5B) and consequently abrogated the inhibitory effect of berberine on oxLDL-induced lipid accumulation (Fig. 5C). These results imply an essential role of LXRα activation in berberine-regulated expression of ABCA1, which may contribute to the suppressive effect of berberine in macrophage foam-cell formation in vitro.

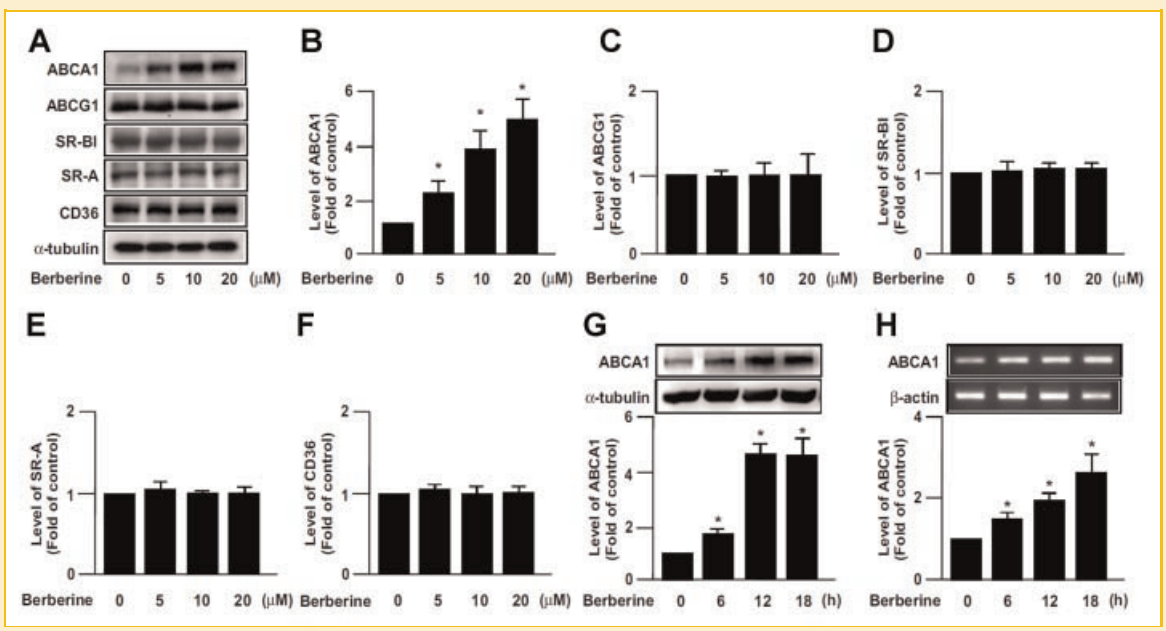

Fig. 2. Berberine upregulates ABCA1 expression in THP-1-derived macrophages. A: THP-1-derived macrophages were treated with berberine (0–20 μM) for 18 h, and the protein expression of ABCA1, ABCG1, SR-B1, SR-A, and CD36 was detected by Western blot analysis. B–F: Protein level in (A) was determined by densitometry. G,H: Macrophages were treated with berberine (10 μM) for 0–18 h, and the protein and mRNA levels of ABCA1 were determined by Western blot analysis and RT-PCR, respectively. Data are mean ± SEM from three independent experiments. $^{*}P < 0.05$ versus control.

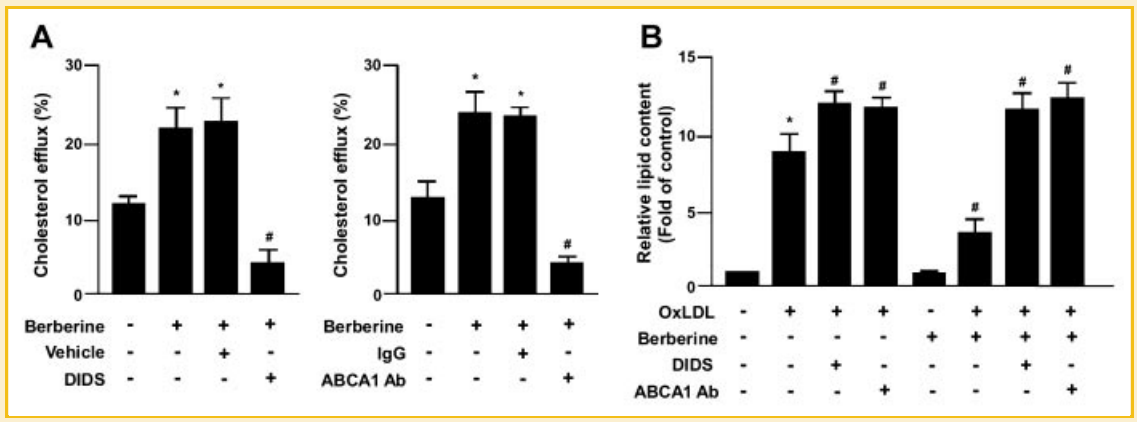

Fig. 3. Inhibition of ABCA1 abrogates the lipid-lowering effect of berberine in macrophages. A: THP-1-derived macrophages were pre-treated with vehicle, DIDS, IgG, or ABCA1 antibody (Ab) for 2 h, incubated with berberine (10 μM) for 12 h, then equilibrated with NBD-cholesterol (1 μg/ml) for an additional 6 h in the presence of berberine. Cholesterol efflux was detected by fluorometry. B: After pre-incubation with DIDS or ABCA1 antibody for 2 h, macrophages were treated with or without berberine (10 μM) for 12 h, then incubated with oxLDL (50 μg/ml) for 18 h. Oil-red O staining and intracellular lipid content was measured. Data are mean ± SEM from three independent experiments. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus oxLDL-treated group.

DISCUSSION

Emerging evidence suggests that berberine is a multifunctional drug that activates various signal pathways and has beneficial effects in in vitro and in vivo models of cardiovascular diseases [Kong et al., 2004; Lee et al., 2006; Lee et al., 2007; Holy et al., 2009]. In addition to its protective effects on endothelial cells and vascular smooth muscle cells [Lee et al., 2006; Holy et al., 2009], berberine can attenuate the inflammatory response in macrophages [Chen et al., 2008; Jeong et al., 2009]. Lipid-laden foam cell accumulation is a critical step in the progression of atherosclerosis because of the augmented inflammation and impaired cholesterol metabolism within vascular walls [de Winther and Hofker, 2000; Kunjathoor et al., 2002; Li and Glass, 2002; Rader and Pure, 2005]. In the current study, we identified a novel suppressive effect of berberine on oxLDL-induced formation of macrophage foam cells. This beneficial effect of berberine on foam cell formation agrees with results by Kong et al. showing that berberine treatment reduced the serum level of cholesterol and retarded the development of atherosclerosis in humans and hamsters [Kong et al., 2004]. According to this observation, we further elucidated the possible mechanisms underlying the berberine-mediated alleviation on foam cell formation.

The intracellular cholesterol homeostasis in macrophages is dynamically regulated by cholesterol uptake and cholesterol efflux, processes that are tightly controlled by SRs and RCTs, respectively [Febbraio et al., 2000; de Winther and Hofker, 2000; Kunjathoor et al., 2002; Ohashi et al., 2005; Cuchel and Rader, 2006; Wang and

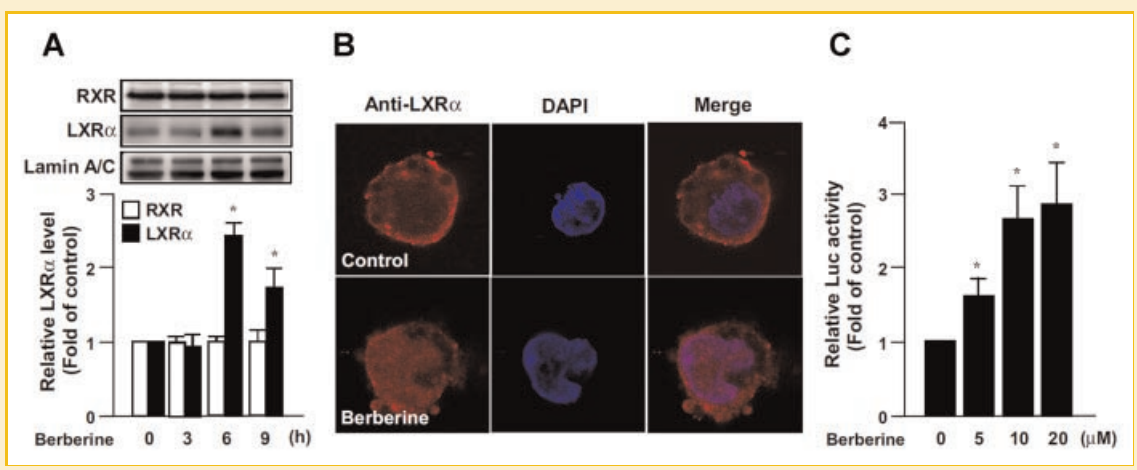

Fig. 4. Berberine induces nuclear translocation and activation of LXRα. THP-1-derived macrophages were incubated with berberine (10 μM) for the indicated times (0–9 h). A: The protein level of LXRα and RXR in nuclear extracts was examined by Western blot analysis. B: Macrophages were treated with berberine (10 μM) for 6 h. After fixation, cells were immunostained with LXRα antibody, and nuclei were stained with DAPI for examination on confocal microscopy. C: Macrophages were transfected with the reporter plasmid 3xLXRE-Luc for 24 h and then treated with indicated concentrations of berberine for another 24 h. Cells were lysed for luciferase (Luc) activity; renilla activity was the internal control. Data are mean ± SEM from three independent experiments. $^{*}P < 0.05$ versus control group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

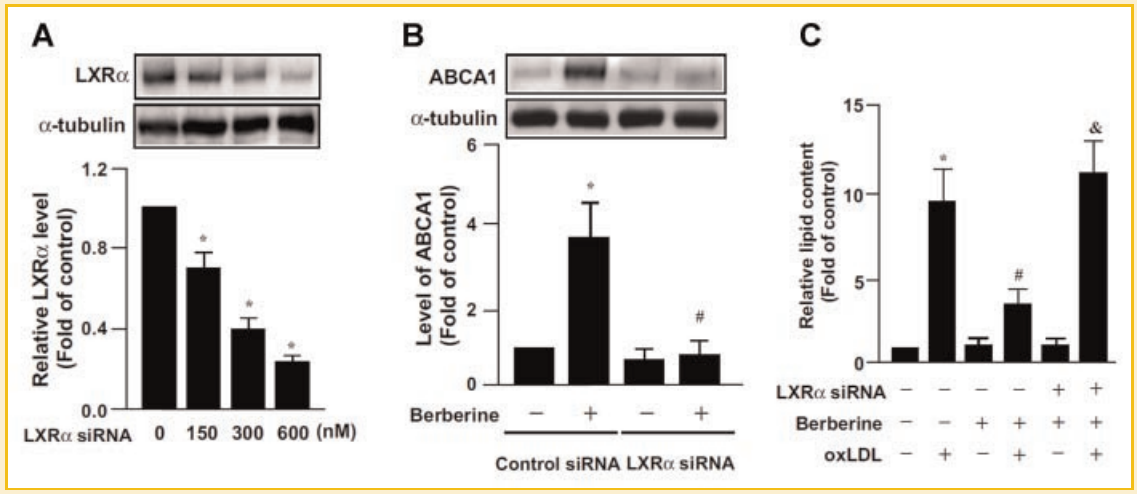

Fig. 5. Knockdown of LXRα expression diminishes the berberine-mediated beneficial effects. A: Macrophages were transfected with various concentrations of LXRα siRNA (150, 300, and 600 nM) for 24 h, and cellular lysates underwent Western blot analysis of the protein level of LXRα. B: Macrophages were incubated with LXRα siRNA (600 nM) for 24 h and then treated with or without berberine (10 μM) for an additional 24 h. The protein level of ABCA1 was examined by Western blot analysis. C: Cells were transfected with LXRα siRNA (600 nM) for 24 h, then berberine (10 μM), oxLDL (50 μg/ml) and/or berberine for an additional 24 h. Intracellular lipid content was measured by alcohol extraction. Data are mean ± SEM from three independent experiments. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus oxLDL-treated group; $^{\&}P < 0.05$ versus berberine/oxLDL-treated group.

Rader, 2007]. Our data showed that berberine treatment did not affect the protein expression of SR-A or CD36 in human THP-1-derived macrophages. In contrast, a recent study by Li et al. [2009] reported that berberine alone significantly induced lipid accumulation in murine macrophages without oxLDL co-treatment and exacerbated the progression of atherosclerosis in apolipoprotein E-deficient mice. The discrepancy in results could be due to differences in cell types and experimental conditions. However, we found that berberine increased cholesterol efflux and upregulated ABCA1 expression in THP-1-derived macrophages. The critical role of ABCA1 in maintaining cholesterol homeostasis in macrophages is well established [Cuchel and Rader, 2006; Wang and Rader, 2007]. Our functional analysis showed that inhibition of ABCA1 activity by use of a pharmacological inhibitor, DIDS, or a specific neutralizing antibody prevented the beneficial effect of berberine against foam cell formation. In view of function, the upregulation of ABCA1 by berberine we observed also likely contributes to the suppression of foam cell formation.

More importantly, we additionally showed that the upregulation of ABCA1 was accompanied by an increase in the nuclear translocation and activation of LXRα, a key transcriptional factor for ABCA1 mRNA expression [Wang and Rader, 2007]. This finding is further supported by promoter activity results showing that berberine increased the promoter activity of 3xLXRE-Luc. Moreover, blockage of LXRα activation by siRNA abrogated ABCA1 induction and therefore berberine's protection against foam cell formation, which indicates that LXRα-mediated transcriptional regulation is required for ABCA1 upregulation and consequent inhibition of lipid accumulation by berberine. Upregulation of ABCA1 has been suggested to be a compensatory mechanism reducing the oxLDL-induced lipid accumulation in foam cells through LXRα activation [Chawla et al., 2001]. In addition, LXRα activation is involved in the upregulation of SR-BI and ABCG1 in adipocytes or macrophages [Kalaany and Mangelsdorf, 2006]. However, our results demonstrated that berberine increased the expression of ABCA1 but not ABCG1 and SR-BI, which implies a differential regulatory mechanism underlying the berberine-mediated LXRα transactivation.

In conclusion, this study demonstrates a unique protective effect of berberine in promoting cholesterol efflux by upregulating ABCA1 expression and transcriptional modulation mediated by LXRα activation, which leads to the suppression of foam cell formation. Berberine may have therapeutic value in inhibiting atherogenesis.

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