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Icariin Inhibits Foam Cell Formation by Down-Regulating the Expression of CD36 and Up-Regulating the Expression of SR-BI

Haitao Yang, Lijie Yan, Peng Qian, Hongyan Duan, Jintao Wu, Bing Li, and Shanling Wang*

Department of Cardiology, Henan Provincial People's Hospital, Zhengzhou 450003, China

ABSTRACT

Icariin is an important pharmacologically active flavonol diglycoside that can inhibit inflammation in lipopolysaccharide (LPS)-stimulated macrophages. However, little is known about the molecular mechanisms underlying the inhibitory effect of Icariin in the formation of foam cells. In this study, macrophages were cultured with LPS and oxidized low-density lipoprotein (oxLDL) in the presence or absence of Icariin. RT-PCR and western blot were used to detect the levels of mRNA and protein expression of CD36, scavenger receptor class B type I (SR-BI) and the phosphorylation of p38MAPK. It was demonstrated that 4 μM or 20 μM Icariin treatment significantly inhibited the cholesterol ester (CE)/total cholesterol (TC) and oxLDL-mediated foam cell formation ($P < 0.05$). The binding of oxLDL to LPS-activated macrophages was also significantly hindered by Icariin ($P < 0.05$). Furthermore, Icariin down-regulated the expression of CD36 in LPS-activated macrophages in a dose-dependent manner and CD36 over-expression restored the inhibitory effect of Icariin on foam cell formation. The phosphorylation of p38MAPK was reduced by Icariin, indicating that Icariin reduced the expression of CD36 through the p38MAPK pathway. In addition, Icariin up-regulated SR-BI protein expression in a dose-dependent manner, and SR-BI gene silencing restored the inhibitory effect of Icariin on foam cell formation. These data demonstrate that Icariin inhibited foam cell formation by down-regulating the expression of CD36 and up-regulating the expression of SR-BI. Therefore, our findings provide a new explanation as to why Icariin could inhibit atherosclerosis. J. Cell. Biochem. 116: 580–588, 2015.

KEY WORDS: ICARIIN; FOAM CELL; CD36; SCAVENGER RECEPTOR CLASS B TYPE I; ATHEROSCLEROSIS

Atherosclerosis (AS), a chronic inflammatory disease, is a serious hazard to human health [Grundtman and Wick, 2011]. Free radical damage plays an important role in the formation and development of AS [Kruth et al., 2005]. In the case of vascular endothelial damage, monocytes are recruited and infiltrate into the artery wall and differentiate into macrophages. Low-density lipoprotein (LDL) in the vascular endothelium is modified into oxidized low-density lipoprotein (oxLDL) and then consumed by the scavenger receptor. This causes intracellular lipid accumulation and ultimately results in the formation of foam cells [Hansson, 2005].

Transformation of macrophages into foam cells is a critical step in the pathogenesis and development of AS. The formation of foam cells is mainly a consequence of the uptake of oxLDL through scavenger receptors on macrophages [Dushkin, 2012]. CD36, an 88,000 MW glycoprotein, is expressed on the surface of endothelial cells and macrophages [Febbraio et al., 2001]. Studies of insects and mice have revealed that CD36 is part of the innate immune system, recognizing molecules present on the surface of certain microbial pathogens, including *Staphylococcus*, mycobacteria and fungi, mediating their clearance by phagocytic cells [Franc et al., 1996; Hoebe et al., 2005; Silverstein et al., 2010]. Previous studies have proven that CD36 is a scavenger receptor, suggesting an important role of CD36 in AS [Park et al., 2012]. Scavenger receptor class B type I (SR-BI), a CD36-related cell surface glycoprotein involved in lipid metabolism [Acton et al., 1996; Rigotti et al., 2003], is a high-density lipoprotein (HDL) receptor expressed on macrophages. It regulates cholesterol uptake in the process of foam cell formation and thereby plays a role in atherosclerosis.

Icariin (2-(4′-methoxylphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone) is an important pharmacologically active flavonol diglycoside isolated from the traditional Chinese herb *Herba epimedii*. It had been

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*Correspondence to: Shanling Wang, Department of Cardiology, Henan Provincial People's Hospital, No. 7 Weiwu Road, Zhengzhou, 450003, China. E-mail: shanglingwang@126.com

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shown that Icariin plays important roles in a multitude of age-dependent disease states, including cancer, bone loss, neurodegenerative disorders, and cardiovascular, mainly due to its anti-oxidant or anti-inflammatory effects [Nian et al., 2009; Wang et al., 2010; Liu et al., 2011]. It has been demonstrated that Icariin can protect DNA from radical-induced oxidative damage [Zhao et al., 2007], and protects erythrocytes against free-radical-induced peroxidation [Liu et al., 2004]. In LPS-stimulated macrophages, Icariin inhibits inflammation by inhibiting tumor necrosis factor-α (TNF-α) production, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression [Chen et al., 2010]. In atherosclerosis, Icariin may also attenuate the enhanced prothrombotic state independently of its lipid-lowering effects [Zhang et al., 2013]. All the above evidence led us to the hypothesis that Icariin can attenuate the development of AS. Therefore, the aim of the present study was to investigate whether Icariin inhibits foam cell formation and to determine the underlying mechanism.

MATERIALS AND METHODS

MATERIALS

If not otherwise mentioned, all substances were purchased from Sigma–Aldrich (St. Louis, MO). Icariin was purchased from Shanghai Ronghe Co. (Shanghai, China). Lipofectamine AMINE 2000, TRIzol, cDNA Synthesis Kit, SYBR® Green PCR Master Mix and RT-PCR kit were obtained from Invitrogen Canada Inc. (Burlington, ON, Canada). Plasmid pIRES2-EGFP and *NheI* and *HindIII* were obtained from Takara (Tokyo, Japan). The BCA protein assay kit, LumiGLo reagent, and NE-PER™ or MEM-PER™ protein extraction reagents were obtained from Pierce Biotechnology (Rockford, IL). Anti-phospho-p38MAPK, anti-p38MAPK, anti-SR-BI, anti-CD36, and anti-β-actin rabbit polyclonal antibodies and the secondary antibodies labeled with horseradish peroxidase (HRP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). LPS, RPMI 1640 medium, fetal bovine serum, and other cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Anisomycin, an activator of p38 MAP kinase, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

PREPARATION OF OXLDL

Human LDL was purified from the fresh plasma of healthy donors by sequential centrifugation, according to Belcher's method, with some modifications [Belcher et al., 1991]. The concentrations of LDL were determined, according to Lowry's method [Lowry et al., 1951] and the LDL at 200 μg/mL was exposed to 20 μM $CuSO_4$ in PBS for oxidation. Following incubation at 37 °C for the indicated periods, the oxidative reactions were stopped with 40 μM butylhydroxytoluene in ethanol. The oxidized LDL (CuoxLDL) was dialyzed against culture medium and sterilely filtered. oxLDL was identified by the behavioral changes in the electrophoretic mobility. The oxLDL was iodinated with $Na^{125}I$, according to Salacinski's method [Salacinski et al., 1981].

CELL CULTURE AND TREATMENTS

The human acute monocytic leukemia cell line THP-1 (ATCC) was cultured in RPMI 1640 media supplemented with 1.0 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 100 μM/L L-ascorbate, 4.5g/L glucose and 10% FCS at 37 °C in a humidified incubator containing 5% CO_2. THP-1 cells were harvested and suspended in fresh medium to a density of 1×10^6 cells per mL. Cells were incubated in the presence of 100 nM phorbol myristate acetate (PMA) (Sigma Chemical Co., St. Louis, MO) for 48–72 h to allow for maximal conversion of the THP-1 monocytes to THP-1 macrophages. After a subpopulation of cells become adherent, morphologic changes of the adherent cells were assessed by phase-contrast microscopy. Macrophages from THP-1 cells were then digested with trypsin, dispensed and incubated according to each experimental design.

FOAM CELL FORMATION ASSAY

Macrophages were incubated with LPS (200 ng/mL) and oxLDL (50 μg/mL) in the presence of the indicated concentration of Icariin for 48 h. Total cholesterol (TC) and cholesterol ester (CE) contents were analyzed by HPLC, according to Liu's [Liu et al., 2012] method with some modifications. Briefly, cells were washed by PBS three times and lysed with a 0.1 N NaOH solution and homogenized on ice for 10 s by ultrasound. The protein concentration was measured using the BCA kit. The organic phase was transferred to clear glass tubes and dried in a vacuum-pump. The samples were dissolved in 100 μL isopropanol-acetonitrile (20:80), followed by an ultrasound water bath for 5 min. Finally, the samples were subjected to HPLC analysis.

Foam cell formation was also determined by the Oil red O-staining assay according to a previous study with minor modifications [Zhou et al., 2013]. Briefly, PBS washed cells were fixed with 5% formalin solution in PBS for 30 min. Then, cells were stained with solution of Oil red O (Primary Cell Co., Ltd., Sapporo, Japan) for 30 min at 37 °C. The Oil red O-stained lipids in macrophage-derived foam cells were morphologically evaluated by microscopy. The lipid accumulation in the foam cells was quantified by isopropanol wash assay. Briefly, after removing the staining solution, the dye retained in the cells was eluted into isopropanol and OD540 was determined using spectrophotometry.

MACROPHAGE BINDING ASSAYS

The ability of ^{125}I-CuoxLDL to bind to macrophages was determined as described previously [Horkko et al., 1999]. To identify the inhibitory effect of Icariin on the binding of ^{125}I-CuoxLDL to macrophages, macrophages were pre-incubated with ^{125}I-CuoxLDL (50 μg/mL), LPS (200 ng/mL), and Icariin (0, 0.8, 4 and 20 μM) at 4 °C for 30 min in serum-free medium. After cells were washed with ice-cold PBS, cell-associated ^{125}I-CuoxLDL was counted using a γ-counter.

TOTAL RNA EXTRACTION AND CDNA SYNTHESIS

Total RNA from macrophages that had been treated with LPS (200 ng/mL), oxLDL (50 μg/mL), and Icariin (0, 0.8, 4 or 20 μM) for 12 h was isolated using TRIzol, as suggested by the manufacturer, and purified with the Qiagen RNeasy kit according to the manufacturer's instructions. Total RNA was reverse transcribed to synthesize first strand cDNA with the Oligo (dT) primer using the cDNA Synthesis Kit.

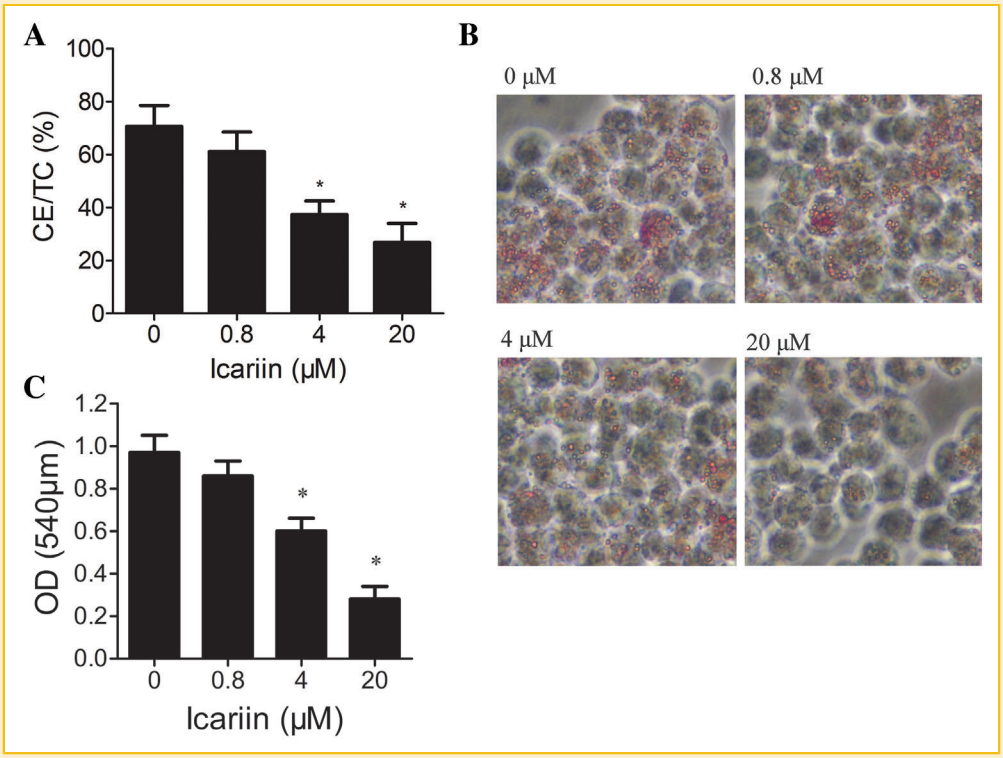

Fig. 1. Icariin inhibits oxLDL-mediated foam cell formation in LPS-activated macrophages. (A) The contents of CE and TC were determined by HPLC. *$P < 0.05$, significantly different from control (0 μM). (B) Foam cell formation was evaluated by oil red O staining (×400). (C) Quantification data of lipid accumulation in the foam cells. After removing the staining solution, the dye retained in the cells was eluted into isopropanol and OD540 was determined using spectrophotometry. Data are mean ± SEM of individual groups (n = 4 per group) from three independent experiments.

REAL-TIME REVERSE TRANSCRIPTION (RT-PCR)

The levels of gene mRNA transcripts were analyzed by one-step quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using the specific primers and SYBR® Green PCR Master Mix and RT-PCR kit, according to the manufacturer's instructions, on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The sequences of primers were 5′-ctccatcctggcctc-gctgt-3′ and 5′-gctgtcaccttcaccgttcc-3′ for CD36; 5′-ggagcaatgatcttgatctt-3′ and 5′-ccttcctgggcatggagtcct-3′ for β-actin; and 5′-agaataagcccatgaccct-gaa-3′ and 5′-tgagctcagcaaataatccgaa-3′ for SR-BI. Briefly, 20 μL reactions containing 50 ng of total RNA, 10 μL 2× SYBR Green PCR Master Mix, 10 U RNase inhibitor, 0.1 mM primers and 6.25 U Multi-Scribe reverse transcriptase were subjected to one cycle of 95 °C for 10 min and then 40 cycles of 95 °C for 15 s, 56°C for 30 s, and 72 °C for 45 s. The amplified products were analyzed by 1.5% agarose gel electrophoresis. For relative quantification, the levels of individual gene mRNA transcripts were first normalized to the control β-actin. Subsequently, the differential expression of these genes was analyzed by the $2^{-\Delta\Delta CT}$ method and expressed as fold changes.

WESTERN BLOT

The harvested cells were lysed with NE-PER™ or MEM-PER™ protein extraction reagent, and their protein concentrations were determined by BCA assay. The protein lysates (40 μg/lane) were cleared by centrifugation at 14,000 × *g*, resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking, the target proteins were probed with 1:500 rabbit anti-CD36, 1:1000 anti-SR-BI, 1:1000 anti-phospho-p38MAPK, anti-p38MAPK, anti-β-actin overnight at 4 °C, and then incubated with HRP-conjugated secondary antibodies for 1 h; the bands were visualized

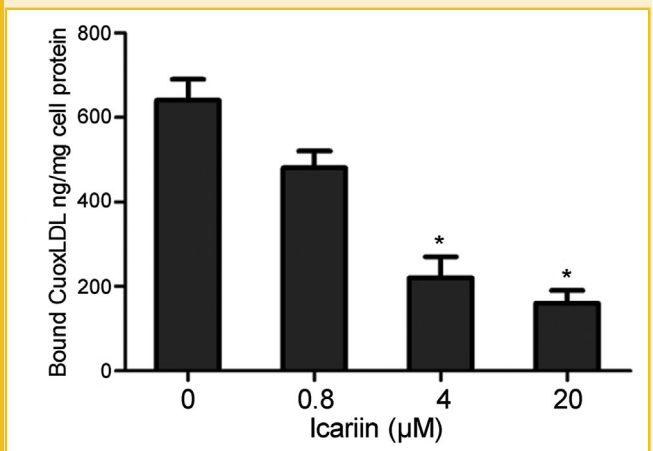

Fig. 2. Icariin inhibits the binding of oxLDL to LPS-activated macrophages. The binding of ^{125}I -CuoxLDL to LPS-activated macrophages was inhibited by different concentrations of Icariin. Data are mean ± SEM of individual groups (n = 4 per group) from two independent experiments. *$P < 0.05$, significantly different from control (0 μM).

using LumiGLo reagent and each protein was quantified in relation to β-actin.

CONSTRUCTION OF CD36 OVER-EXPRESSION PLASMID AND TRANSFECTION

Construction of CD36 expression plasmids and transfection was performed as previously described [Hsu and Turvey, 2013]. Briefly, the CD36 cDNA fragments were cloned into the plasmid pIRES2-EGFP with the restriction enzymes *NheI* and *HindIII*. The recombinant constructs were designated pIRES2-EGFP-CD36. The pIRES2-EGFP-CD36 plasmids were mixed with DMEM medium without serum to a total volume 50 μL and incubated at room temperature for 5 min. Lipofectamine 2000 (2.0 μL) was mixed with DMEM medium without serum, and was then mixed with the above plasmid mixture and incubated at room temperature for 20 min. The final mixtures were incubated with the 70–90% confluence THP-1 macrophages for 6 h. Then, the old medium was removed and new DMEM medium with 10% calf serum was added. After 72 h, the cells were cultured with neomycin (G418) (200 g/L) for 4 weeks to obtain the constitutively-expressed cell line. The efficiency of transfection was determined by the expression of enhanced green fluorescence protein (EGFP), measured with microscopy using GFP filter (Nikon, Tokyo, Japan). CE/TC contents in macrophages were measured in cells which had been treated with LPS (200 ng/mL), oxLDL (50 μg/mL), and Icariin (4 μM) for 48 h.

SR-BI GENE SILENCING

An si-genome SMARTpool (Shanghai Genetimes) consisting of three unique 27mer siRNA duplexes and control siRNA was used to knock down SR-BI expression levels, as previously described [Liu et al., 2012]. In brief, cultured THP-1 cells were transfected with 20 μmol/L SR-BI siRNA using Lipofectamine 2000 1 h prior to the addition of LPS (200 ng/mL), oxLDL (50 μg/mL), and Icariin (4 μM), according to the manufacture's protocol. CE/TC contents in macrophages were measured in cells which had been treated with LPS, oxLDL, and Icariin for 48 h.

STATISTICAL ANALYSES

To compare values among multiple groups, one-way analysis of variance (ANOVA) was applied. Data are presented as mean ± SEM, except where otherwise indicated. For comparison of mean values between two groups, the unpaired *t*-test was used. Statistical significance was assumed if $P < 0.05$.

RESULTS

ICARIIN INHIBITS THE FORMATION OF FOAM CELLS

During the pathogenic process of atherosclerosis, monocytes can infiltrate into artery walls and functionally differentiate into macrophages. Following the binding of oxidized and modified LDL, macrophages differentiate into foam cells [Naito, 2008]. Foam cells play integral roles in every stage of the process of atherosclerosis [Bobryshev, 2006] and express various markers and inflammatory mediators [Hansson et al., 2006]. Icariin, a compound isolated from *Herba epimedii*, possesses multiple pharmacological activities. Previous studies have revealed that Icariin can be considered a potential drug for the treatment of inflammatory diseases [Chen et al., 2010]. In this study, we evaluated whether Icariin could affect foam cell formation. Here, LPS-activated macrophages treated with oxLDL were incubated with or without Icariin, and the TC and CE content-markers of foam cell formation were measured using HPLC. As shown in Figure 1A, 0.8 μM Icariin treatment down-regulated the rate of CE/TC, but no significant difference was observed as compared to the control group. After 4 μM or 20 μM Icariin treatment, the level of CE/TC was significantly decreased ($P < 0.05$, Fig. 1A). The results of oil red O staining also demonstrated that foam cell formation was hindered by Icariin (Fig. 1B). The quantified data also showed that the lipid loading in the foam cells were significantly reduced in cell with 4 μM or 20 μM Icariin treatment (Fig. 1C). These data suggested that Icariin decreases the differentiation of LPS-activated macrophages into foam cells.

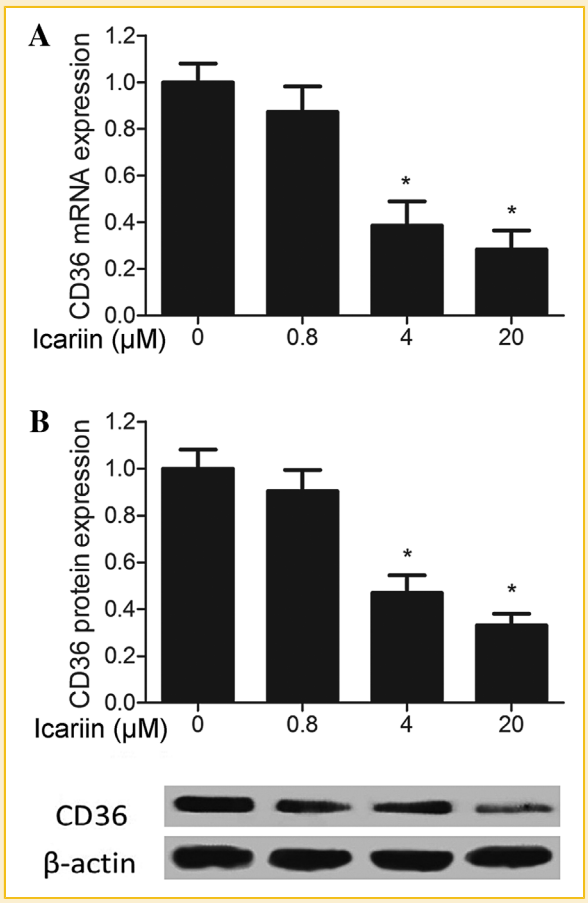

Fig. 3. Icariin inhibits expression of CD36 mRNA and protein in LPS-activated macrophages. (A) The expression of CD36 mRNA was determined by RT-PCR. Data are expressed as the relative expression levels normalized to β-actin. (B) The expression of CD36 protein was determined by western blot assays. Data are expressed as the relative expression levels normalized to β-actin ($n = 4$). *$P < 0.05$, significantly different from control (0 μM).

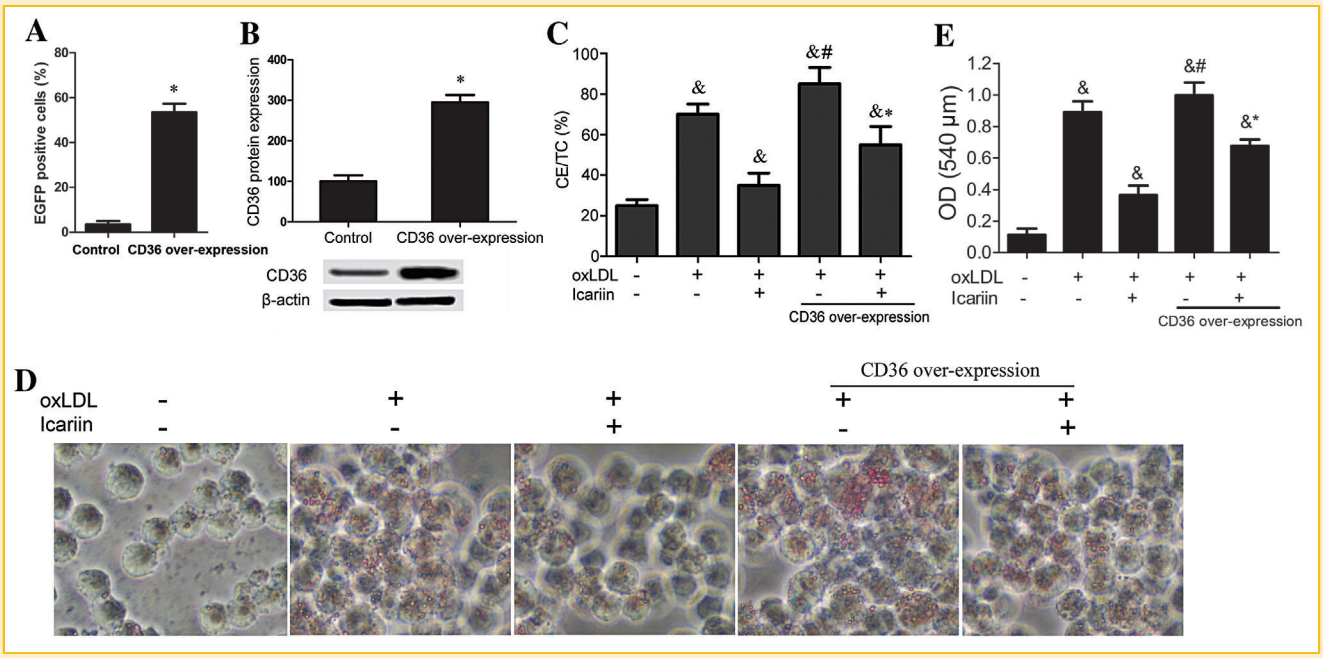

Fig. 4. CD36 gene over-expression increases CE/TC production in LPS-activated macrophages. (A) The efficiency of transfection was determined by the expression of EGFP, measured with microscopy using GFP filter (Nikon, Tokyo, Japan) ($n = 4$). $^{*}P < 0.05$, significantly different from normal control. (B) Cell lysates were prepared from these cells, proteins were resolved by SDS-PAGE and CD36 expression was detected by Western blotting. Data are expressed as the relative ratio (%), normalized to β-actin ($n = 4$). (C) CE/TC contents were measured by HPLC 48 h after stimulation ($n = 4$). $^{\&}P < 0.05$, compared with column 1. $^{\#}P < 0.05$, column 4 versus column 2. $^{*}P < 0.05$, column 5 versus column 3. (D) Foam cell formation was evaluated by oil red O staining (×400). (E) Quantification data of lipid accumulation in the foam cells. After removing the staining solution, the dye retained in the cells was eluted into isopropanol and OD540 was determined using spectrophotometry. Data are mean ± SEM of individual groups ($n = 4$ per group) from three independent experiments. $^{\&}P < 0.05$, compared with column 1. $^{\#}P < 0.05$, column 4 versus column 2. $^{*}P < 0.05$, column 5 versus column 3.

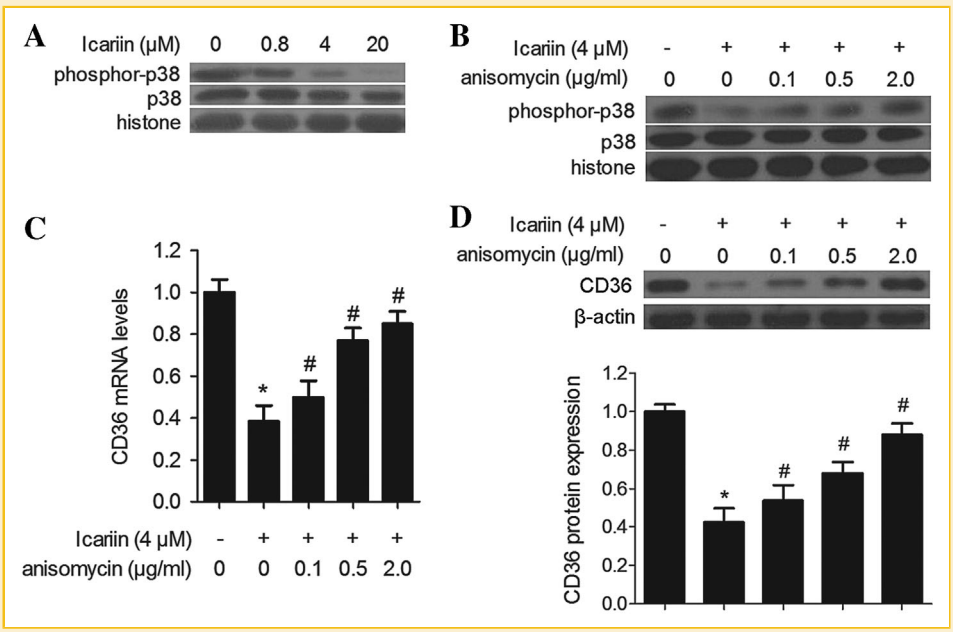

Fig. 5. Icariin down-regulated CD36 expression partly through inhibition of p38MAPK pathway. (A) Cells treated with indicated concentration of Icariin were lysed and the phosphorylation of p38 were determined by western blot. Data are expressed as the relative expression levels normalized to total histone. (B) Cells pre-treated with 4 μM Icariin were incubated with indicated concentration of anisomycin for 40 min. The phosphorylation of p38 were determined by western blot. Data are expressed as the relative expression levels normalized to total histone. The expression of CD36 were detected by RT-PCR (C) and western blot (D). Data are expressed as the relative expression levels normalized to β-actin ($n = 4$). $^{*}P < 0.05$, significantly different from normal control (0 μM Icariin, 0 μg/mL anisomycin). $^{\#}P < 0.05$, significantly different from treatment control (4 μM Icariin, 0 μg/mL anisomycin).

ICARIIN INHIBITS THE BINDING OF OXLDL TO LPS-ACTIVATED MACROPHAGES

OxLDL bound to the macrophages and resulted in the formation of foam cells. To identify the binding of oxLDL with macrophages and the influences exerted by Icariin, we applied ^{125}I-CuoxLDL and different concentrations of Icariin during the culture of LPS-activated macrophages. The levels of bound ^{125}I-CuoxLDL in macrophages were significantly suppressed by treatment with Icariin at 4 μM and 20 μM (Fig. 2). These data suggest that Icariin inhibited the formation of foam cells by inhibiting the binding of oxLDL to LPS-activated macrophages. The above data indicated that Icariin was a potential drug to inhibit foamy macrophage formation and atherosclerosis-related pathogenesis.

ICARIIN INHIBITS THE EXPRESSION OF CD36 IN LPS-ACTIVATED MACROPHAGES

Scavenger receptors are integral membrane proteins that mediate the endocytosis of modified lipoproteins. CD36, a member of the class B scavenger receptor family of cell surface proteins, was shown to be implicated in oxLDL uptake and foam cell formation [Calvo et al., 1998; Dhaliwal and Steinbrecher, 1999; Nicholson et al., 2000]. On macrophages, CD36 interacts with oxidized oxLDL and triggers a signaling response that is pro-inflammatory and pro-atherogenic. In order to clarify whether CD36 is involved in the Icariin-reduced foam cell formation, the levels of CD36 mRNA transcripts and cellular proteins were analyzed by RT-PCR and western blot assays, respectively. The 0.8 μM Icariin treatment down-regulated CD36 mRNA (Fig. 3A) and protein (Fig. 3B) levels, but no significant difference was observed. CD36 mRNA (Fig. 3A) and protein (Fig. 3B) levels were both significantly decreased after 4 μM Icariin treatment, and were further decreased after 20 μM Icariin treatment. This data indicated that Icariin down-regulated CD36 mRNA and protein expression in a dose-dependent manner (Fig. 3).

CD36 GENE OVER-EXPRESSION RESTORES THE INHIBITORY EFFECT OF ICARIIN ON FOAM CELL FORMATION

To further elucidate the role of CD36 in the process by which Icariin reduces foam cell formation, the CD36 gene was over-expressed using the constructed expression plasmid and transfected into macrophages. The efficiency of transfection reached 53.4% (Fig. 4A). Western blotting was used to detect the suppression of CD36 expression. As shown in Figure 4B, CD36 expression levels were significantly enhanced. We further measured the CE/TC content to assess foam cell formation. As shown in Figure 4C, CE/TC content increased to 84% in the CD36 over-expressed group treated without Icariin, compared to 71% in the group without CD36 over-expression. In the other two groups which were treated with Icariin, the CE/TC content significantly increased to 54% in the CD36 over-expressed group, compared to 34% in the group without CD36 over-expression. The oil red O staining assay also revealed the same trend (Fig. 4D). These data suggest that CD36 gene over-expression restored the inhibitory effect of Icariin on foam cell formation and also demonstrate a direct link between Icariin and foam cell formation.

ICARIIN DOWN-REGULATED CD36 EXPRESSION THROUGH INHIBITION OF P38MAPK PATHWAY

It had been reported previously that CD36 expression in macrophages could be modulated through the inhibition of p38 MAPK phosphorylation [Min et al., 2013]. In this study, LPS-activated macrophages were treated with varying concentrations of Icariin and the levels of total p38MAPK and phosphor-p38MAPK were characterized by western blot assays (Fig. 5A). Clearly, the expression level of phosphor-p38MAPK was significantly down-regulated in LPS-activated macrophages following treatment with Icariin. The results suggested that Icariin down-regulated the expression of CD36, likely by suppressing activation of the p38MAPK pathway. To further confirm the role of p38MAPK pathway in the function of Icariin on CD36 expression, anisomycin, a well characterized potent activator of p38 MAP kinase [Rolli et al., 1999; Li et al., 2003], was involved in this study. We first determined whether the inhibitory effect of Icariin on CD36 expression could be abrogated by a prior treatment with anisomycin. As expected,

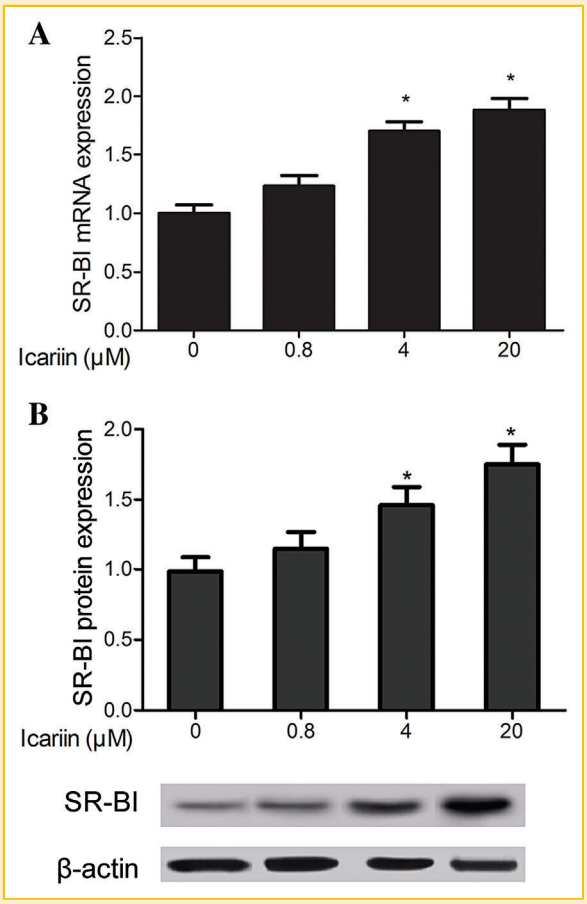

Fig. 6. Icariin up-regulates expression of SR-BI mRNA and protein in LPS-activated macrophages. (A) The expression of SR-BI mRNA was determined by RT-PCR. Data are expressed as the relative expression levels normalized to β-actin. (B) The expression of SR-BI protein was determined by western blot assays. Data are expressed as the relative expression levels normalized to β-actin. Values are expressed as the mean ± SEM ($n = 4$). *$P < 0.05$, significantly different from control (0 μM).

anisomycin treatment led to the activation of p38 as determined by western blot (Fig. 5B). Meanwhile, anisomycin alleviated the inhibitory effect of Icariin on CD36 expression in a dose-dependent fashion (Fig. 5C,D). This data indicated that Icariin down-regulated CD36 expression through inhibition of p38MAPK pathway.

ICARIIN PROMOTES SR-BI EXPRESSION IN LPS-ACTIVATED MACROPHAGES

The deposition of lipids in vessel walls and the development of atheromas are thought to be opposed by the removal of cholesterol and its transportation back to the liver for secretion, a process that is known as reverse cholesterol transport [Mindham and Mayes, 1991]. Efflux of cell-free cholesterol from peripheral cells into acceptor particles is the first step of this process. Although the mechanisms of cellular cholesterol efflux remain to be clarified, certain classes of HDL are believed to play a key role in this process [Jian et al., 1998]. SR-BI is an HDL receptor which mediates selective uptake of HDL cholesterol esters into cells. Previous studies have shown that SR-BI mediates potentially important atheroprotective signaling in endothelial cells and macrophages induced by HDL [Zhang et al., 2003; Van Eck et al., 2005; Huby et al., 2006]. SR-BI-deficient mice have increased HDL cholesterol levels, but increased atherosclerosis [Huby et al., 2006], suggesting that SR-BI expression is inversely related to plasma levels of HDL cholesterol and the development of atherosclerosis. Adenoviral expression of the P279S variant in SR-BI-deficient mice led to a reduction in the cholesterol ester uptake capacity of hepatocytes in these mice when compared to wild-type SR-BI, and the cholesterol efflux from monocyte–macrophages obtained from carriers of the P279S variant was lower, as compared to non-carriers [Vergeer et al., 2011]. In line with this concept, SR-BI has been shown to play an anti-atherogenic role in mice [Huszar et al., 2000; Braun et al., 2002]. Here, RT-PCR and western blot analysis were carried out to determine whether Icariin promotes the expression of the SR-BI protein. It was demonstrated that Icariin up-regulated SR-BI mRNA and protein expression in a dose-dependent manner (Fig. 6), indicating that Icariin promotes SR-BI expression at the transcriptional level in LPS-activated macrophages.

SR-BI GENE SILENCING RESTORES THE INHIBITORY EFFECT OF ICARIIN ON FOAM CELL FORMATION

To further elucidate the role of SR-BI in the process of foam cell formation and the inhibition of this by Icariin, SR-BI gene expression was knocked down using specific siRNA in macrophages. Western blotting was used to detect the suppression of SR-BI expression. As shown in Figure 7A, the expression of SR-BI was reduced to 34% in SR-BI specific siRNA transfected macrophages, this was significant different from cells in mock group or those transfected with a scrambled sequence. We further measured the CE/TC content to

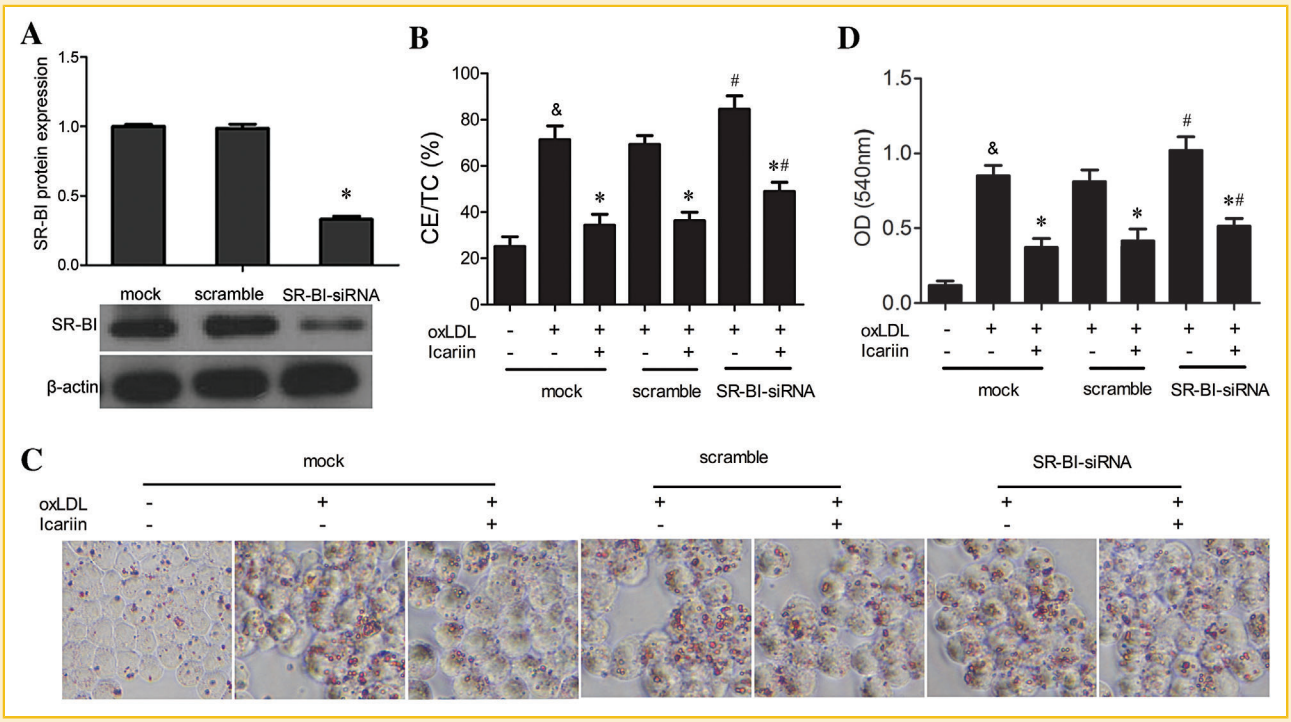

Fig. 7. SR-BI specific siRNA transfection increases CE/TC production in LPS-activated macrophages. (A) Cell lysates were prepared from these cells, proteins were resolved by SDS-PAGE and SR-BI expression was detected by western blotting. Data are expressed as the relative normalized to β-actin. $^{*}P < 0.05$, significantly different from mock group ($n = 4$). (B) CE/TC contents were measured by HPLC 48 h after stimulation. $^{\&}P < 0.05$, column 2 versus column 1. $^{*}P < 0.05$, column 3 versus column 2, column 5 versus column 4, column 7 versus column 6. $^{\#}P < 0.05$, column 4 and 6 versus column 2, column 5 and 7 versus column 3 ($n = 4$). (C) Foam cell formation was evaluated by oil red O staining (×400). (D) Quantification data of lipid accumulation in the foam cells. After removing the staining solution, the dye retained in the cells was eluted into isopropanol and OD540 was determined using spectrophotometry. $^{\&}P < 0.05$, column 2 versus column 1. $^{*}P < 0.05$, column 3 versus column 2, column 5 versus column 4, column 7 versus column 6. $^{\#}P < 0.05$, column 4 and 6 versus column 2, column 5 and 7 versus column 3 ($n = 4$).

analyze the effect of SR-BI gene knockdown on foam cell formation. As shown in Figure 7B, in the three groups treated without Icariin, the CE/TC content increased to 84% in the SR-BI silenced group, compared to 71% in the mock group and 69% in the scramble group; in the other three groups treated with Icariin, the CE/TC content significantly increased to 51% in the SR-BI silenced group, compared to 34% in the mock group and 36% in the scramble group. A similar result was also obtained from the oil-red O staining assay (Fig. 7C,D). These data suggest that SR-BI gene silencing restored the inhibitory effect of Icariin on foam cell formation and also demonstrate a direct link between Icariin and foam cell formation.

CONCLUSION

These data indicated a potential role for Icariin in inhibiting foam cell formation and atherosclerosis, through down-regulating the expression of CD36 and up-regulating the expression of SR-BI, respectively. Further studies should be aimed at in vivo studies to shed more light on the therapy of atherosclerosis and its associated diseases.

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