

Protective effects of icariin on human vascular endothelial cells induced by oxidized low-density lipoprotein via modulating caspase-3 and Bcl-2

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Abstract. Icariin belongs to the family of flavonoids that is extracted from *Epimedium brevicornum* Maxim, and exhibits antioxidative, antitumorigenic, antiosteoporotic, immunoregulatory and antiatherosclerotic properties. To understand the mechanisms underlying the antiatherosclerotic properties of icariin, the present study investigated the effects of icariin on human vascular endothelial cells (HUVECs) following treatment with oxidized low-density lipoprotein (ox-LDL). Thus, following pretreatment with icariin at four various concentrations (0, 10, 20 and 40 μ M), HUVECs were stimulated with ox-LDL (100 μ g/ml). The viability of cells was evaluated via an MTT assay and flow cytometry was performed to assess apoptosis. Additionally, the protein and mRNA expression levels of apoptosis regulator Bcl-2 (Bcl-2) and caspase-3 were determined by western blotting and reverse transcription-quantitative polymerase chain reaction. The findings of the present study indicated that icariin prevented injury and apoptosis in HUVECs following ox-LDL treatment, in particular via the regulation of protein and mRNA expression levels of Bcl-2 and caspase-3.

Introduction

Atherosclerosis (AS) is chronic vascular inflammation (1) involving lumen narrowing and rigidity due to cholesterol and lipid accumulation (2,3). Additionally, AS is associated with vascular endothelial damage, following which low-density

lipoprotein (LDL) enters the subendothelial layer where it is oxidized (ox-LDL) and subsequently consumed by scavenger receptors. Consequently, monocytes are recruited and infiltrate the artery wall, where they differentiate into macrophages (4,5). Thus, the ox-LDL-associated damage to vascular endothelial cells (VECs) is directly associated with the initiation and development of AS (6,7).

Traditional Chinese Medicine has widely employed *Epimedium brevicornum* Maxim in 'tonifying kidney and strengthening bone' in China, Korea and Japan (8-10). Icariin ($C_{33}H_{40}O_{15}$; Fig. 1) is a pharmacologically active flavonoid extracted from *E. brevicornum* Maxim (11,12), with numerous pharmacological properties, including antiosteoporosis (13), antitumor (14), immunoregulation (15), anti-inflammation (5) and antioxidation (16). Icariin is additionally used to treat cardiovascular diseases and exhibits anti-atherosclerotic properties (17-21) that are associated with its protective effects on endothelial cells (17); however, the underlying mechanisms require further investigation. Thus, the present study analyzed the effects of icariin on ox-LDL-induced injury and apoptosis in human (HUVECs) by evaluating cell viability apoptosis and its associated genes and proteins, including caspase-3 and apoptosis regulator Bcl-2 (Bcl-2), in injured human HUVECs with or without treatment with icariin.

Materials and methods

Cell culture and treatments. HUVECs were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 12 h (37°C, 5% CO₂) in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Hangzhou Sijiqing Bioengineering Material Co., Ltd., Hangzhou, China), 1% penicillin and 1% streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Following pretreatment with 0, 10, 20 and 40 μ M icariin (Sichuan Weike Biotechnology Co., Ltd., Chengdu, China) for 24 h at 37°C, cells were

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treated for 24 h with 100 $\mu\text{g/ml}$ ox-LDL (Guangzhou Yiyuan Biological Technology Co., Ltd., Guangzhou, China).

Viability of cells. Based on a previous report (17), the cells were maintained for 24 h in serum-free DMEM to achieve cell cycle synchronization prior to their treatments with icariin and ox-LDL. Following pretreatment with icariin and ox-LDL, MTT reagent (0.5 mg/ml) was added to the cells at a density of 1×10^4 cells/well and they were incubated for 4 h at 37°C. Subsequently, the precipitate was dissolved in 150 μl dimethyl sulfoxide, and the optical density of the supernatant was measured at a wavelength of 490 nm.

Apoptosis. In the present study, the apoptotic ability was evaluated using an Annexin V fluorescein isothiocyanate (FITC) kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing China), FACSCalibur (BD Biosciences, San Jose, CA, USA) and ModFit LT V3.3.11 software (Verity Software House Inc., Topsham, ME, USA). Cells were washed with PBS and centrifuged for 5 min at 800 \times g at 4°C, and the treated cells were resuspended in 200 μl 1X Annexin binding buffer and harvested. Subsequently, cells were stained with 5 μl propidium iodide (PI; Sigma-Aldrich; Merck KGaA) and 2.5 μl Annexin V/FITC, and protected from light for 15 min at 37°C.

Western blotting. HUVECs were seeded into 6-well plates at a density of 1×10^4 cells/well, treated as aforementioned, harvested and lysed for 30 min in ice-cold radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). Following centrifugation for 20 min at 13,000 \times g and 4°C, the supernatants were analyzed using a bicinchoninic acid assay. Equal amounts of protein samples (50 μg) were loaded onto a 15% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes, which were blocked with 5% fat-free milk for 1 h at room temperature. At 4°C membranes were incubated with rabbit anti-human monoclonal antibodies against caspase-3 (1:1,000; cat. no. ab23021; Abcam, Cambridge, UK), anti-Bcl-2 (1:1,000; cat. no. ab47482; Abcam) or anti-GAPDH (1:1,000; Sigma-Aldrich; Merck KGaA; cat. no. sab4300645) overnight, followed by horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:5,000; cat. no. sc45101; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at ambient temperature. The bands were visualized by enhanced chemiluminescence/X-ray films (GE Healthcare, Little Chalfont, UK) and were analyzed using ImageJ version 1.46 (National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from HUVECs with TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 5 μg total RNA was reverse transcribed using a PrimeScript RT Master Mix kit (Takara Biotechnology Co., Ltd., Dalian, China), and RT-qPCR was performed using SYBR® Premix Ex Taq (Beijing Transgen Biotech Co., Ltd., Beijing, China) and the following program: Denaturing at 95°C for 10 sec, annealing at 60°C for 15 sec and extension at 72°C for 30 sec (40 cycles). GAPDH was used as a housekeeping gene. The following oligonucleotide primers were used: Caspase-3 forward, 5'-GTGGAATTGATGCGTGATG-3' and

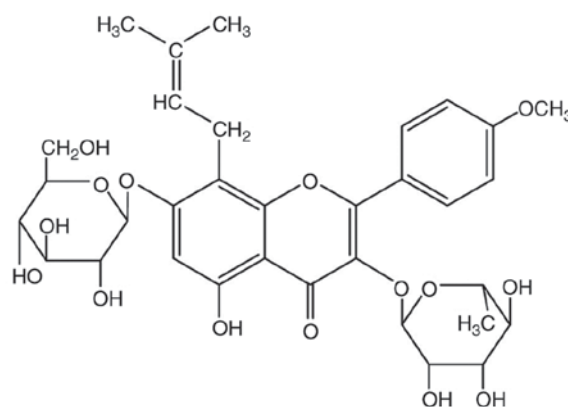


Figure 1. Chemical structure of icariin.

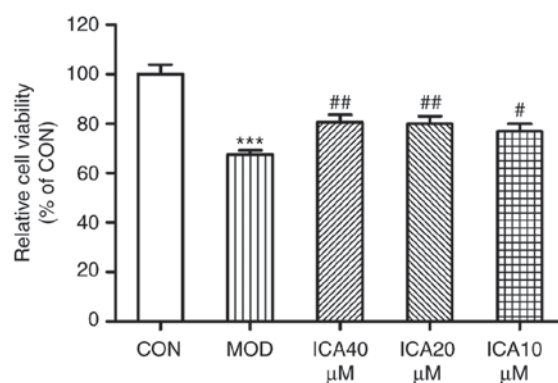


Figure 2. Protective effects of icariin in human vascular endothelial cells. Cells were treated for 24 h with icariin (0, 10, 20 and 40 μM) followed by oxidized low-density lipoprotein (100 $\mu\text{g/ml}$). Based on the MTT assay, cell viability was significantly promoted by pretreatment with icariin. *** $P < 0.001$ vs. CON; * $P < 0.05$, ** $P < 0.01$ vs. MOD. CON, control; ICA, icariin-treated group; MOD, oxidized low-density lipoprotein-simulated group.

reverse, 5'-GGAATCTGTTTCTTTGCATG-3'; Bcl-2 forward, 5'-GGTGCCACCTGTGGTCCACCT-3' and reverse, 5'-CTTCACTTGTGGCCAGATAGG-3'; and GAPDH forward, 5'-GTTACCAGGGCTGCCTTCTC-3' and reverse, 5'-GATGGTGATGGGTTTCCCGT-3'. Relative quantification was calculated using the $2^{-\Delta\Delta\text{Cq}}$ method (22) and the results were normalized to those of GAPDH.

Statistical analysis. The SPSS 19.0 program (IBM, Corp., Armonk, NY, USA) was used. Each independent experiment was performed in triplicate. One-way analysis of variance followed by a Tukey's post-hoc analysis was used, and the data are presented as the mean \pm standard error. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Protective effects of icariin. To investigate whether icariin exerts a protective effect against injury, the present study analyzed the viability of HUVECs treated with ox-LDL via an MTT assay. Treatment with ox-LDL significantly decreased the viability of HUVECs compared with control cells (Fig. 2), while icariin mitigated this decrease. These findings suggested

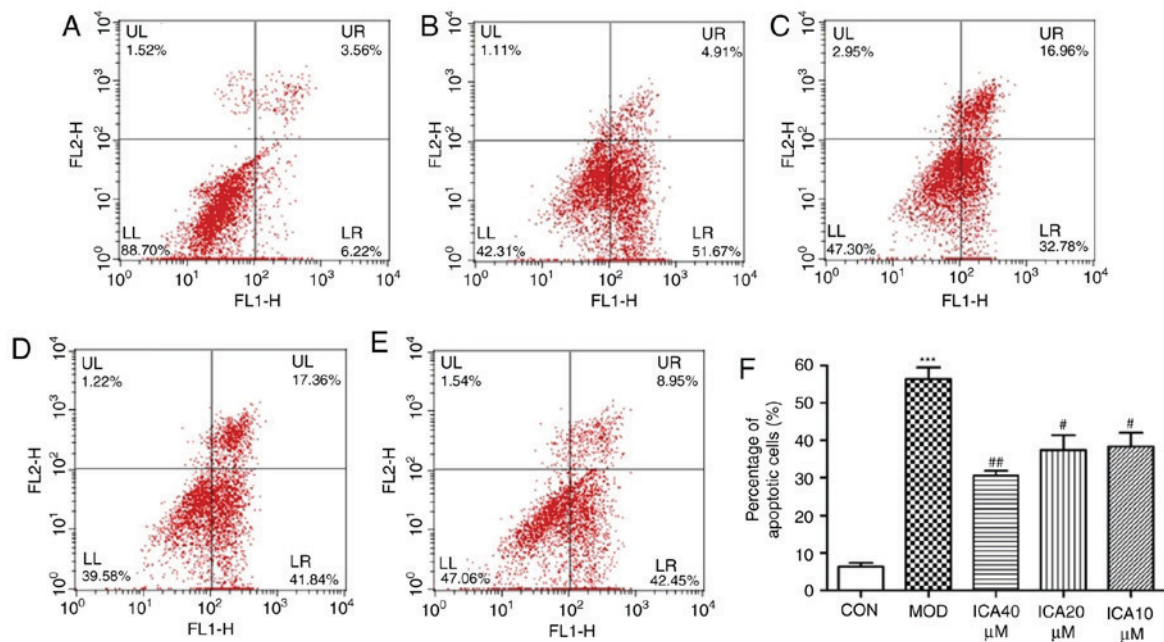


Figure 3. Antiapoptotic effects of icariin on HUVECs. Following 24 h pretreatment with icariin at different doses (0, 10, 20 and 40 μ M), cells were stimulated with ox-LDL (100 μ g/ml), and flow cytometry was used to determine the rate of apoptosis. (A) Representative results of control cells. (B) Representative results of HUVECs stimulated with ox-LDL. Representative results of icariin at (C) 10, (D) 20 and (E) 40 μ M in HUVECs stimulated with ox-LDL. (F) Percentage of early apoptotic cells following the indicated treatments. *** P <0.001 vs. CON; # P <0.05, ## P <0.01 vs. MOD. HUVECs, human vascular endothelial cells; ICA, icariin-treated group; ox-LDL, oxidized low-density lipoprotein; MOD, ox-LDL-simulated group.

that icariin may have exerted protective effects against injury within HUVECs stimulated with ox-LDL.

Antiapoptotic effects of icariin. To determine the effect of icariin on cellular apoptosis, Annexin-V and PI double staining was performed. The present study investigated the apoptosis rate of HUVECs treated with ox-LDL using flow cytometry. The apoptosis rate significantly increased when HUVECs were treated with ox-LDL compared with the control group (Fig. 3). Additionally, pretreatment with icariin significantly mitigated this effect, the improvement was more significant at 40 μ M icariin compared with the other assayed concentrations. These findings indicated that icariin markedly inhibited apoptosis in HUVECs stimulated with ox-LDL.

Regulation of caspase-3 and Bcl-2 in HUVECs by icariin. To investigate the mechanism underlying the protective effects of icariin on ox-LDL-induced cellular apoptosis, the relative gene and protein expression levels of Bcl-2 and caspase-3 were quantified via RT-qPCR and western blot analysis, respectively. Treatment of HUVECs with ox-LDL significantly increased caspase-3 and decreased Bcl-2 expression at the protein (Fig. 4) and mRNA level (Fig. 5). Conversely, icariin pretreatment significantly suppressed these alterations. The results indicated that icariin exerts antiapoptotic effects by downregulating caspase-3 mRNA and protein expression levels while upregulating those of Bcl-2.

Discussion

Previous reports have demonstrated the harmful effects of ox-LDL, which induced the apoptosis of endothelial cells (23). Furthermore, ox-LDL is involved in the pathogenesis of

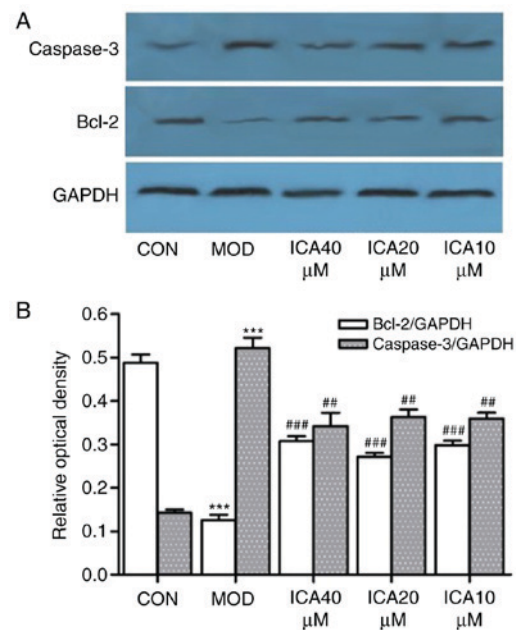


Figure 4. Icariin-induced regulation of caspase-3 and Bcl-2 protein expression in human vascular endothelial cells. Following pretreatment with icariin (0, 10, 20 and 40 μ M), cells were treated with ox-LDL (100 μ g/ml) for 24 h, and the protein expression levels of caspase-3 and Bcl-2 were detected by western blot analysis; as a loading control, GAPDH was used. (A) Expression of Bcl-2 and caspase-3. (B) Ratio of Bcl-2 and caspase-3 expression levels to control levels. *** P <0.001 vs. CON; # P <0.05, ### P <0.01 vs. respective MOD group. Bcl-2, apoptosis regulator Bcl-2; CON, control; ICA, icariin-treated group; ox-LDL, oxidized low-density lipoprotein; MOD, ox-LDL-simulated group.

AS by injuring the vascular endothelium (24,25). Previous studies have suggested that ox-LDL may directly target VECs and induce apoptosis via the mitochondrial apoptotic

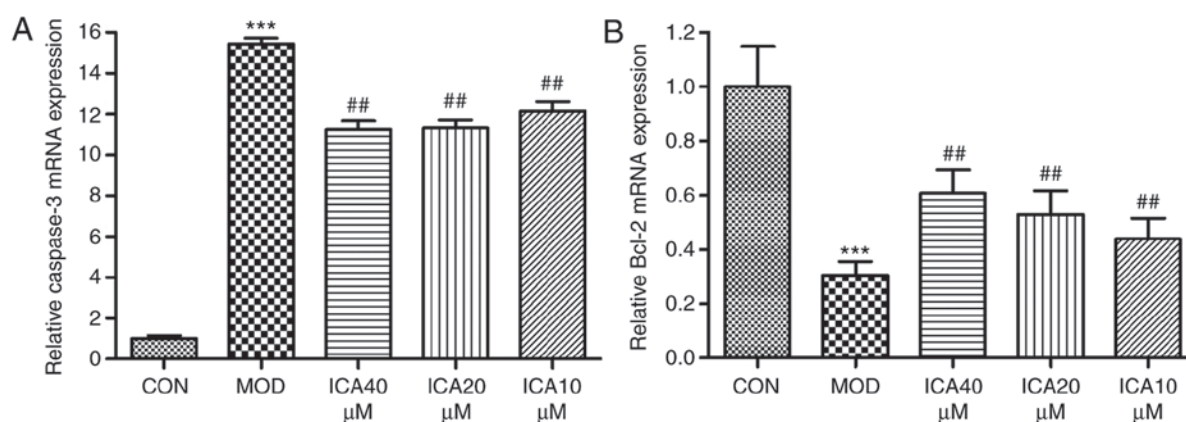


Figure 5. Regulation of Bcl-2 and caspase-3 gene expression in human vascular endothelial cells by icariin. Following pretreatment with icariin (0, 10, 20 and 40 μ M), cells were treated for 24 h with ox-LDL (100 μ g/ml). Subsequently, the expression levels of Bcl-2 and caspase-3 mRNA were determined by reverse transcription-quantitative polymerase chain reaction; as a loading control, GAPDH was used. (A) Caspase-3 mRNA expression and the ratio to the control level. (B) Bcl-2 mRNA expression and the ratio to the control level. *** P <0.001 vs. CON; ** P <0.01 vs. MOD. Bcl-2, apoptosis regulator Bcl-2; CON, controls; ICA, icariin-treated group; ox-LDL, oxidized low-density lipoprotein; MOD, ox-LDL-simulated group.

pathways (26-29). In addition, ox-LDL promotes the recruitment of monocytes and reactive oxygen species (30-32) by upregulating (32,33) and binding to the lectin-like ox-LDL receptor (23) on VECs. Caspases have been suggested to be associated with the signaling pathways underlying ox-LDL-induced apoptosis (23). Treatment with ox-LDL has been reported to result in the activation of caspase-9, thus resulting in the activation of caspase-3, the major effector caspase responsible for the destruction of various substrates, including the proteins involved in DNA repair, mRNA splicing, and DNA replication (34).

Therefore, injury to endothelial cells in the subendothelial space of the arterial wall is a critical pathological cascade in the occurrence of AS. To the best of our knowledge, the present study is the first to demonstrate that icariin may significantly suppress injury induced by ox-LDL in HUVECs. The effects of icariin were associated with increased apoptosis. Treatment with ox-LDL notably reduced HUVEC viability, increasing the apoptosis rate. Icariin significantly reversed ox-LDL-mediated effects in HUVECs.

In order to elucidate the mechanism involved in the protective influences of icariin in HUVECs, Bcl-2 and caspase-3 at the gene and protein expression levels were investigated. It has been reported that the caspase cascade served a pivotal role of in apoptosis. Caspase-3 is activated during the final step of the proapoptotic signaling pathway, while the suppression of caspase activity attenuates injury and apoptosis in HUVECs (35). The Bcl-2 family of proteins is considered to be an important family of apoptosis regulators, and include anti- and pro-apoptotic molecules (36,37). The results of the present study indicated significantly reduced Bcl-2 mRNA and protein levels within ox-LDL-treated HUVECs compared with the control. Conversely, the forced expression of Bcl-2 mRNA and protein attenuated HUVEC apoptosis caused by ox-LDL, and suppressed caspase-3 activity.

In summary, the present study demonstrated that icariin inhibited HUVEC damage and apoptosis induced by ox-LDL. The antiapoptotic effects were associated with the downregulation of caspase-3 and upregulation of Bcl-2. The results of the present study provided additional evidence that icariin may

prevent the development of AS; however, further investigation into the biological activity of icariin, including its effects on vascular smooth muscle cells or foam cells, is required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YH performed the experiments and wrote the manuscript, and KL, YZ performed the cell study. HL designed the study, performed bibliographic research, drafted the manuscript and provided comments. LR and ZF designed the study, analyzed the data and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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