

Icariin inhibits oxidized low-density lipoprotein-induced proliferation of vascular smooth muscle cells by suppressing activation of extracellular signal-regulated kinase 1/2 and expression of proliferating cell nuclear antigen

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Abstract. Icariin, a flavonoid isolated from the traditional Chinese herbal medicine *Epimedium brevicornum* Maxim, has been shown to possess anti-inflammatory, anti-oxidant and anti-atherosclerotic activities *in vivo* and *in vitro*. The aim of the present study was to investigate the effects of icariin on oxidized low-density lipoprotein (ox-LDL)-induced proliferation of vascular smooth muscle cells (VSMCs) and the possible underlying mechanism. VSMCs were cultured and pre-treated with various concentrations of icariin (0, 10, 20 or 40 μ M) prior to stimulation by ox-LDL (50 μ g/ml). Cell proliferation was evaluated by an MTT assay. Flow cytometry was used to study the influence of icariin on the cell cycle. Proliferating cell nuclear antigen (PCNA) expression and phosphorylation levels of extracellular signal-regulated kinase (ERK)1/2 were detected by western blot analysis. The results indicated that icariin significantly inhibited ox-LDL-induced proliferation of VSMCs and phosphorylation of ERK1/2. Furthermore, icariin also blocked the ox-LDL-induced cell-cycle progression at G1/S-interphase and downregulated the expression of PCNA in VSMCs. In conclusion, the present study indicated for the first time that icariin reduced the amount of ox-LDL-induced proliferation of VSMCs through suppression of PCNA expression and inactivation of ERK1/2.

Introduction

Atherosclerosis is a chronic vascular inflammatory disease, characterized by narrowing and rigidity of the lumen as a result of cholesterol and lipid accumulation (1,2). Abnormal proliferation of intimal vascular smooth muscle cells (VSMCs) leads to intimal thickening of the aorta, and has an important role in initiation and amplification of atherogenesis (3). Oxidized low-density lipoprotein (ox-LDL) is a mitogen in VSMCs and stimulates the proliferation of VSMCs and activation of the extracellular signal-regulated protein kinase (ERK)1/2 signaling pathway. Therefore, the ox-LDL-induced proliferation of VSMCs in the intima of the arterial wall is considered to be an important factor in atherosclerotic plaque development (4).

Epimedium brevicornum Maxim, a traditional Chinese herbal medicine, has been widely used for tonifying kidneys and strengthening bone for thousands of years in China, Korea and Japan (5-7). Icariin (C₃₃H₄₀O₁₅; molecular weight, 676.67; Fig. 1), a flavonoid isolated from *Epimedium brevicornum* Maxim, is considered as the main pharmacological active constituent (8,9) and has been reported to possess various pharmacological effects, including anti-inflammatory, anti-osteoporosis (10), anti-tumor (11), immunoregulatory (12) and anti-oxidative actions (13). In addition, icariin has been shown to have beneficial effects on cardiovascular diseases such as atherosclerosis (14,15). However, the potential mechanisms of action of icariin against atherosclerosis have remained to be fully elucidated. In view of this, the present study was designed to elucidate whether icariin can attenuate the initiation and progression of atherosclerosis. The effects of icariin on ox-LDL-induced proliferation of VSMCs were assessed, and the results indicated that they are mediated via suppression of cell-cycle regulatory protein proliferating cell nuclear antigen (PCNA) and deactivation of ERK1/2.

Materials and methods

Cell culture. Human aortic vascular smooth muscle cells (HA-VSMCs) were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in 100-mm

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dishes in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sijiqing Bioengineering Material Co., Ltd. Hangzhou, China) and 1% penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA) at 37°C in a humidified atmosphere containing 5% CO₂.

Cell viability assay. VSMCs in the logarithmic growth phase were seeded into 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h at 37°C in an atmosphere containing 5% CO₂. After pre-treatment with the indicated concentrations of icariin (purity, >98%; Vic's Biological Technology Co., Ltd, Sichuan, China; 0, 10, 20 or 40 μ M) for 24 h prior to stimulation with oxidized low-density lipoprotein (ox-LDL; Yiyuan Biotechnologies Co., Ltd, Guangzhou, China; 50 μ g/ml) for the indicated times (24 or 48 h). Subsequently, the medium was discarded and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) solution was added at a final concentration of 0.5 mg/ml, followed by incubation for 4 h at 37°C. The MTT solution was carefully removed and 150 μ l dimethyl sulfoxide (Sigma-Aldrich) was added to each well followed by a 15-min incubation. The absorbance of each well was measured using a microplate reader (Multiskan MK3; Thermo Fisher Scientific, Inc.) with a reference wave length of 490 nm. The initial absorbance at 0 h, prior to icariin treatment, was also measured.

Cell cycle analysis. VSMCs in the logarithmic growth phase were seeded into six-well plates at a density of 1×10^4 cells per well and then incubated for 24 h at 37°C in an atmosphere containing 5% CO₂. After pre-treatment with the indicated concentrations of icariin (0, 10, 20 or 40 μ M) for 24 h, cells were incubated with or without ox-LDL (50 μ g/ml) for a further 24 h. The cells were trypsinized, collected and washed twice with ice-cold phosphate-buffered saline (PBS) prior to fixing in 70% cold ethanol at 4°C overnight. Next, the fixed cells were re-suspended in PBS containing 100 μ g/ml RNase A (Sigma-Aldrich) and 50 μ g/ml propidium iodide (PI; Sigma-Aldrich) for 30 min at room temperature. Cells were then analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The percentages of cells in G0/G1, S and G2/M phases were determined using ModFit LT V3.3.11 software (Verity Software House Inc., Topsham, ME, USA).

Western blot analysis. VSMCs in the logarithmic growth phase were seeded into six-well plates for incubation for 24 h at 37°C in an atmosphere containing 5% CO₂. After pre-treatment with the indicated concentrations of icariin (0, 10, 20 or 40 μ M) for 24 h, the cells were incubated with or without ox-LDL (50 μ g/ml) for another 24 h. Subsequently, VSMCs were scraped in ice-cold PBS and lysed in cold lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). After centrifugation at $13,000 \times g$, the supernatant (total protein extract) was separated and quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein samples (50 μ g) were loaded onto 10% SDS-PAGE gels (Applygen Technologies, Inc., Beijing, China) and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The membranes

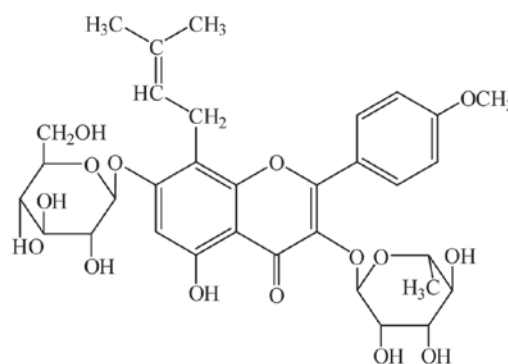


Figure 1. Chemical structure of icariin (C₃₃H₄₀O₁₅; molecular weight, 676.67).

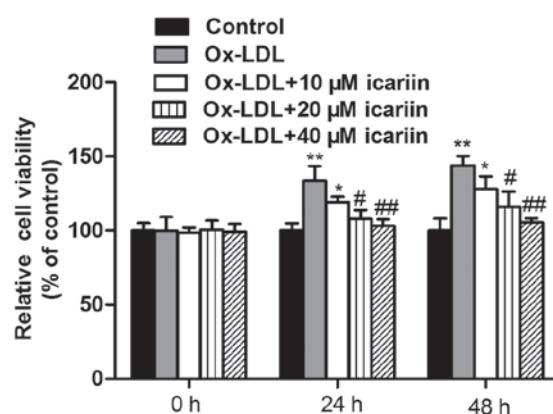


Figure 2. Effects of icariin on ox-LDL-induced proliferation of VSMCs. VSMCs were pre-treated for 24 h with different concentrations of icariin and then exposed to ox-LDL (50 μ g/ml) for 24 or 48 h. Cell viability was assessed by the MTT method. Icariin pre-treatment significantly increased the cell viability. Values are expressed as the mean \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. the control group, # $P < 0.05$ and ## $P < 0.01$ vs. the ox-LDL-simulated group. VSMC, vascular smooth muscle cell; ox-LDL, oxidized low-density lipoprotein.

were blocked with 5% fat-free milk in Tris-buffered saline containing 0.05% Tween 20 (TBST; Zhongtian Jingwei Technologies, Inc., Beijing, China) and incubated with the primary rabbit anti-human monoclonal antibodies against ERK1/2 (dilution, 1:1,000; Abcam, Cambridge, MA, USA; cat. no. ab36911), anti-phosphorylated-ERK1/2 (dilution, 1:1,000; Abcam; cat. no. 50011) or anti-PCNA (dilution, 1:1,000; Abcam; cat. no. ab92552) or anti-GAPDH (dilution, 1:1,000; Sigma-Aldrich; cat. no. sab4300645) at 4°C overnight. Following incubation, the membranes were washed three times in TBST for 15 min and the membranes were incubated with horseradish-peroxidase-labeled goat anti-rabbit secondary antibody for 1 h at room temperature (dilution, 1:5,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-45101). Following three further washes in TBST, the protein expression levels were visualized using the enhanced chemiluminescence kit, BeyoECL Plus (Beyotime Institute of Biotechnology), images of the blots were captured on X-ray films (GE Healthcare, Little Chalfont, UK) and were analyzed using ImageJ version 1.46 (National Institutes of Health, Bethesda, MD, USA). GAPDH was used as the protein loading control.

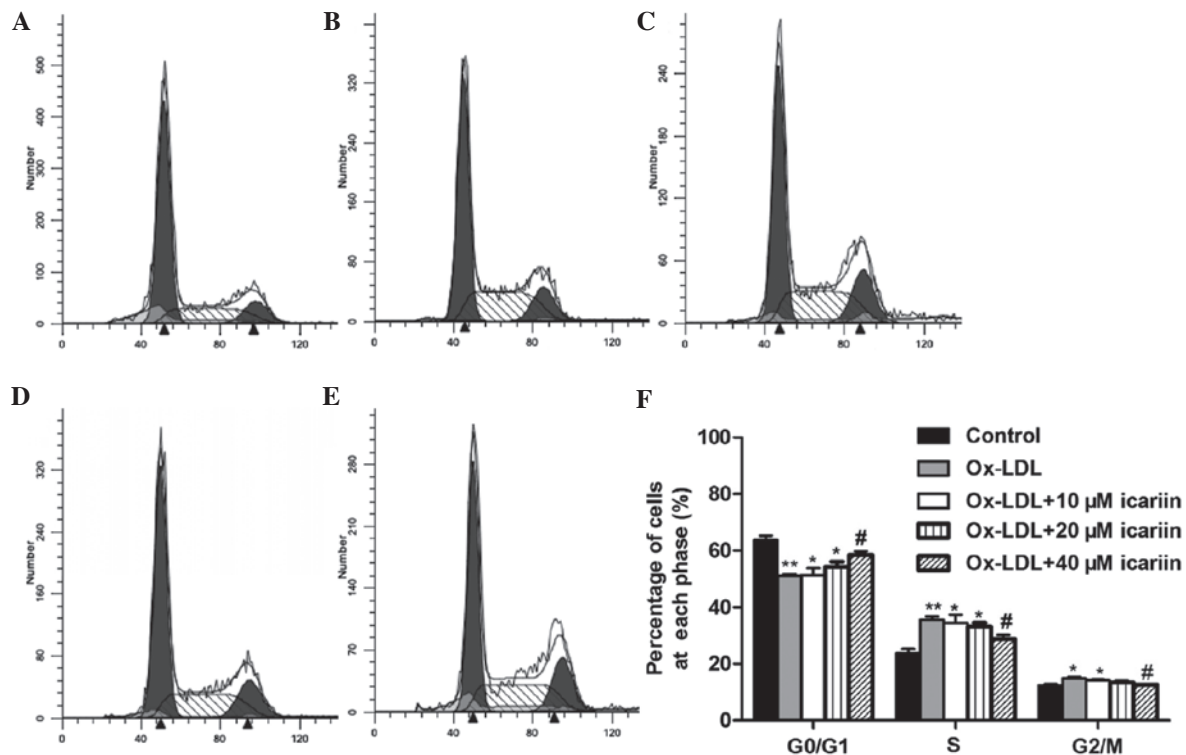


Figure 3. Effects of icariin on the cell cycle of VSMCs induced by ox-LDL. VSMCs were pre-treated with various concentrations of icariin (0, 10, 20, 40 µM) and then incubated with ox-LDL (50 µg/ml) for another 24 h. The cell cycle distribution was determined by assessing the individual nuclear DNA content reflected by the fluorescence intensity of incorporated propidium iodide. Flow cytometric analysis of (A) VSMCs without any treatment (control), (B) VSMCs induced by ox-LDL and (C-E) VSMCs induced by ox-LDL following pre-treatment with icariin at 10, 20 or 40 µM, respectively. (F) Percentages of cells in G0/G1, S and G2/M phases after the indicated treatments. Representative flow cytometry graphs are shown and results are expressed as the mean \pm standard deviation from three independent experiments. * P <0.05 and ** P <0.01 vs. the control group, # P <0.05 vs. the ox-LDL-simulated group. VSMC, vascular smooth muscle cell; ox-LDL, oxidized low-density lipoprotein.

Statistical analysis. Statistical analysis was performed using the SPSS 17.0 statistical package (SPSS, Inc., Chicago, IL, USA). Values are expressed as the mean \pm standard deviation. One-way analysis of variance was applied for multiple comparisons and the least significant difference test was applied for intra-group comparison. P <0.05 was considered to indicate a statistically significant difference.

Results

Icariin inhibits ox-LDL-induced VSMC proliferation. To evaluate the effects of icariin on the proliferation of VSMCs induced by ox-LDL, the cell viability was assessed using an MTT assay. As shown in Fig. 2, exposure of the cells to ox-LDL for 24 or 48 h significantly increased the cell viability compared with that in the control group. However, icariin inhibited ox-LDL-induced VSMC proliferation in a concentration-dependent manner.

Icariin reduces ox-LDL-induced cell cycle progression and PCNA expression. To clarify the effect of icariin on cell-cycle regulation, the cell cycle distribution of ox-LDL-induced VSMCs was assessed using flow cytometry. As shown in Fig. 3, treatment with ox-LDL markedly increased the percentage of VSMCs in S and G2/M phases and correspondingly decreased the percentage of cells in G0/G1 phase. However, pre-treatment with icariin significantly reversed these effects in a concentration-dependent manner. To assess whether the effects of icariin on the cell cycle were associated with the

expression of PCNA, western blot analysis was performed. As shown in Fig. 4, the protein expression of PCNA was markedly increased following ox-LDL treatment, which was inhibited by icariin in a dose-dependent manner. The western blot results were in line with the findings of the cell cycle analysis, as ox-LDL enhanced the population of cells in S phase, which was accompanied an increased expression of PCNA, while icariin pre-treatment was able to inhibit these effects in a concentration-dependent manner. These results suggested that icariin inhibits ox-LDL-induced VSMC proliferation by blocking cell cycle progression.

Icariin inhibits ox-LDL-induced phosphorylation of ERK1/2. To demonstrate whether icariin inhibited ox-LDL-induced VSMC proliferation by inhibiting the activation of ERK1/2, the phosphorylation levels of ERK1/2 were examined. As shown in Fig. 5, ox-LDL and icariin had no effect on the levels of total ERK1/2. VSMCs incubated with ox-LDL for 24 h showed markedly enhanced ERK1/2 phosphorylation, which was significantly and dose-dependently inhibited by icariin pre-treatment. These results suggested that icariin may reduce ox-LDL-induced proliferation of VSMCs, at least in part, via inhibition of ox-LDL-induced ERK1/2 phosphorylation.

Discussion

Atherosclerosis is a chronic vascular inflammatory disease (16). It is characterized by the formation of atherosclerotic plaques

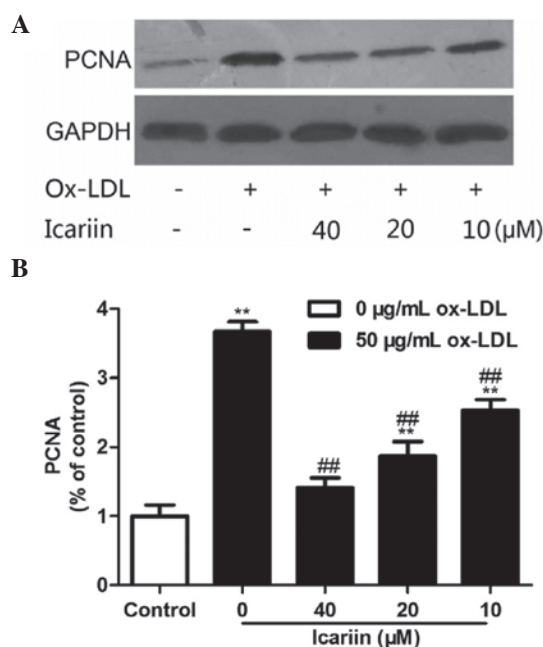


Figure 4. Effects of icariin on ox-LDL-induced PCNA expression in VSMCs. VSMCs were pre-treated with various concentrations of icariin (0, 10, 20 or 40 μ M), and then incubated with ox-LDL (50 μ g/ml) for another 24 h. Proteins were extracted from the cells and assessed by western blot analysis. GAPDH served as the loading control. (A) Western blot assay for PCNA expression. Blots are representative of three independent experiments. (B) PCNA expression was quantified by densitometric analysis and shown as the ratio of the control. Values are expressed as the mean \pm standard deviation from three independent experiments. ** P <0.01 vs. the control group, ## P <0.01 vs. the ox-LDL-simulated group. VSMC, vascular smooth muscle cell; ox-LDL, oxidized low-density lipoprotein; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

consisting of foam cells, leukocytes, platelets, inflamed smooth muscle cells and endothelial cells (17). It is increasingly recognized that ox-LDL has a critical role in the promotion of atherosclerosis initiation, progression and plaque destabilization (18). Several studies have indicated that ox-LDL can stimulate the proliferation of VSMCs and the activation of ERKs (4,19). Therefore, VSMC proliferation induced by ox-LDL in the intima of the arterial wall is thought to have a critical role in the development of atherosclerotic lesions (4). However, inhibition of VSMC proliferation represents a potentially important therapeutic strategy for the prevention and treatment of atherosclerosis (20). The present study showed that ox-LDL induced VSMC proliferation and provided the first evidence that icariin inhibited ox-LDL-stimulated VSMC proliferation in a concentration-dependent manner.

The cell cycle is a highly regulated process that involves a complex cascade of events to regulate cell proliferation (21-23). It comprises three distinct phases: The G0/G1 phase, the DNA synthesis-associated S phase and the G2/M phase (23,24). Under normal conditions, VSMCs proliferate at low rates, largely remaining in the G0/G1 phase of the cell cycle. Following proliferative stimulation, VSMCs re-enter the cell cycle (3,25). In the present study, flow-cytometric analysis indicated that ox-LDL treatment promoted the proliferation of VSMCs and increased the S-phase population with a simultaneous decrease in the G0/G1-phase population, while pre-treatment with icariin significantly reversed these effects.

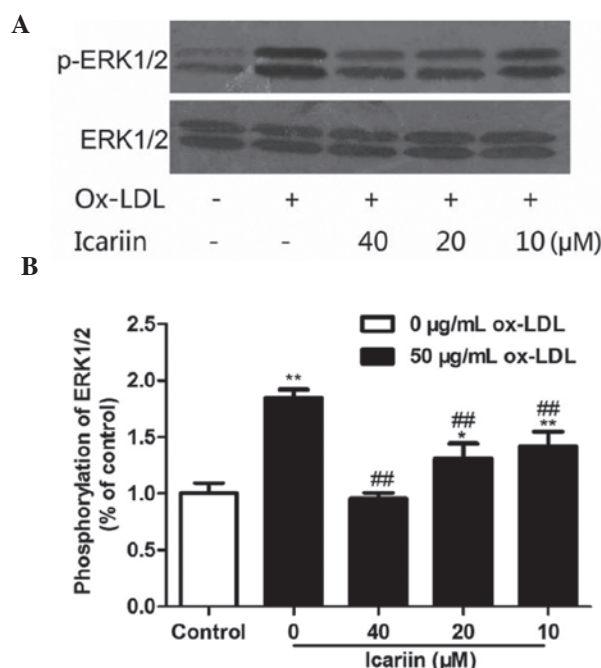


Figure 5. Effects of icariin on ERK1/2 phosphorylation in VSMCs. VSMCs were pre-treated with various concentrations of icariin (0, 10, 20 or 40 μ M) and then exposed to ox-LDL (50 μ g/ml) for another 24 h. Proteins were extracted from the cells and assessed by western blot analysis. Total ERK1/2 served as a loading control. (A) Western blot assays for ERK1/2 and p-ERK1/2 expression. Blots are representative of three independent experiments. (B) The p-ERK1/2/ERK1/2 expression ratio was quantified by densitometric analysis and shown as the ratio of the control. Values are expressed as the mean \pm standard deviation from three independent experiments. * P <0.05 and ** P <0.01 vs. the control group, ## P <0.01 vs. the ox-LDL-simulated group. VSMC, vascular smooth muscle cell; ox-LDL, oxidized low-density lipoprotein; p-ERK, phosphorylated extracellular signal-regulated kinase.

The results suggested that icariin may reduce ox-LDL-induced proliferation of VSMCs by inhibiting their transition from G0/G1 phase to S phase.

PCNA is involved in a number of essential cellular processes, including DNA repair, DNA replication and cell-cycle regulation (26), and is regulated by a variety of mechanisms that act at the transcriptional as well as the post-transcriptional level (27). PCNA is required for G0/G1-to-S phase transition and its synthesis is tightly regulated during the cell cycle (4). The present study found that the percentage of VSMCs in S and G2/M phase increased after treatment with ox-LDL, which was inhibited by pre-treatment with icariin. These findings were in line with the effects of ox-LDL and icariin on the protein expression of PCNA: Ox-LDL treatment enhanced the expression of PCNA, while icariin dose-dependently inhibited these increases. It is therefore likely that icariin inhibited ox-LDL-induced VSMC proliferation through inhibition of PCNA expression.

ERK is a widely expressed protein kinase and an intracellular signaling molecule that is involved in cell proliferation (28). Previous studies have indicated that ox-LDL induces VSMC proliferation through activation of the ERK pathway (19,29,30). In line with these results, the present study revealed that the phosphorylation of ERK1/2 in VSMCs was enhanced by ox-LDL, which was inhibited by pre-treatment with icariin. These results suggested that icariin significantly

inhibited ox-LDL-induced proliferation of VSMCs by blocking cell-cycle progression, partly via inhibiting the ox-LDL-induced activation of ERK1/2.

In conclusion, the present study demonstrated that icariin inhibited the proliferation of VSMCs stimulated by ox-LDL via decreasing the S-phase population of the cell cycle. Ox-LDL-induced phosphorylation of ERK1/2 and the expression of PCNA were also suppressed by icariin. These findings suggested that icariin may inhibit ox-LDL-induced proliferation of VSMCs by inactivating the ERK1/2 signaling pathway and by suppressing the expression of PCNA. Icariin may therefore be able to reduce the development of atherosclerosis.

Acknowledgements

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