Icariin Derivative Inhibits Inflammation through Suppression of p38 Mitogen-Activated Protein Kinase and Nuclear Factor-κB Pathways

Shao-Rui Chen, a Xiang-Zhen Xu, a Yu-Hua Wang, a Jian-Wen Chen, a Suo-Wen Xu, a Lian-Quan Gu, b and Pei-Qing Lu a

a Laborotary of Pharmacology and Toxicology, Sun Yat-Sen University; and b Laboratory of Bioorganic and Medicinal Chemistry, School of Pharmaceutical Sciences, Sun Yat-Sen University; 132 East Wai-huan Road, College City of Guangzhou, Guangzhou 510006, China.

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In this study we investigated the anti-inflammatory effects of an icariin derivative (3,5-dihydroxy-4’-methoxy-6’,6”-dimethyl-1”,4”,5”-dihydropyranon[2””,3””’,7,8]-flavone). We found that this icariin derivative inhibits tumor necrosis factor-α (TNF-α) production, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA expression, and protein expression in lipopolysaccharide (LPS) stimulated RAW264.7 macrophages. It also alleviates paw edema induced by carrageenan in mice. To clarify the molecular mechanisms underlying these anti-inflammatory effects, we examined the effects of this compound on the phosphorylation of mitogen-activated protein kinase (MAPK), phosphorylation of inhibitory kappaBalpha (IκBα), and nuclear translocation of p65 subunit of nuclear factor-κB (NF-κB) and found it suppresses the activation of p38 MAPK and inhibits translocation of NF-κB p65 to the nucleus through decreasing the phosphorylation of IκBα. As a result of these properties, this icariin derivative can be considered as a potential drug for inflammatory diseases.

Key words icariin derivative; tumor necrosis factor-α; inducible nitric oxide synthase; cyclooxygenase-2; mitogen-activated protein kinase; nuclear factor-κB

Inflammation is a host response to external/internal challenge that leads to the release of a large amount of inflammatory mediators. Prolonged or overactivated inflammation contribute to the pathogenesis of many diseases such as bronchitis, rheumatoid arthritis, and chronic nephritis.1—3 Macrophages play a critical role in immune reactions and serve as an essential interface between innate and adaptive immunity.4 Lipopolysaccharide (LPS), which is one of the most potent microbial initiators of inflammation, activates several signaling pathways in macrophages by acting on toll-like receptor (TLR)4 to induce the expression of inflammatory gene and the release of mediators/cytokines such as interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α), and nitric oxide (NO), all of which are involved in the pathogenesis of many diseases.5,6 The cytokine TNF-α is a soluble homotrimer of 17kDa protein subunits secreted primarily by monocytes, macrophages, and T cells in response to endotoxin or other stimuli.7 It is known as a proinflammatory cytokine that possesses a multitude of biological activities linked to septic shock, inflammation, cachexia and cell death.8 Inducible NO synthase (iNOS) expression is significantly induced by LPS or cytokines in a variety of immune cells.9 It catalyzes the oxidative deamination of L-arginine to produce NO, a potential pro-inflammation mediator. Overproduction of NO appears to be linked to tissue damage and organ dysfunction.10 Cyclooxygenase-2 (COX-2) is another pivotal enzyme in the inflammation process. COX-2 is barely detectable under normal physiological conditions, however, it can be rapidly induced in macrophages by stimuli including cytokines, endotoxin and growth factors. Activated COX-2 converts arachidonic acid to prostanoids (including prostaglandins, prostacyclin and thromboxanes) causing pain, edema, and vasodilation in the inflammation site.11,12 In these events, the inhibition of excess macrophage activities and the attenuation of the expression of TNF-α, NO and COX-2 should serve as the basis for the potential development of anti-inflammatory therapy.

The molecular mechanism of LPS-induced macrophage activation has been intensively investigated. Various kinases are involved, including mitogen-activated protein kinases (MAPKs).13 MAPKs are a highly conserved family of serine/threonine kinases including extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun NH2-terminal kinase (JNK). They are all important signaling molecules in the control of cellular responses to outside stimuli.14 It has been demonstrated that the phosphorylation of MAPKs is a critical component of the production of NO and pro-inflammation cytokines in activated macrophages.15,16 Nuclear factor-κB (NF-κB) is an important transcription factor complex that regulates the expression of many genes that code for mediators involved in immune and inflammatory responses, e.g. iNOS, TNF-α and COX-2.17,18 Therefore, NF-κB has become a logical target for new types of anti-inflammatory treatment.19

Epimedium are known as traditional herbal medicines used in the treatment of chronic nephritis, osteoporosis, asthma, cardiovascular problems, and hepatitis in Eastern Asia.20,21 It has been confirmed through pharmacological assays that these immunomodulatory effects are based on the presence of active constituents.22—24 Icariin, which is an important bioactive constituent in Epimedium, has demonstrated anti-inflammatory ability.25 In our preliminary experiments, we tested the anti-inflammatory effect of a series of icariin derivatives as well as icariin in LPS-activated macrophages and found that one compound (3,5-dihydroxy-4’-methoxy-6”,6”-dimethyl-1”,4”,5”-dihydropyranon[2””,3””’,7,8]-flavone) (Fig. 1) had a more potent anti-inflammatory action than the others (data not shown). Therefore, we examined the anti-inflammatory effects of this icariin derivative in vivo and in vitro, and investigated the underlying mechanism of its action.
Concentrations were quantified. The icariin derivative was dissolved in dimethyl sulfoxide (DMSO) after 4 h incubation in culture dishes. Test compound and LPS administration were carried out under serum-free conditions. The TNF-α influence in culture was determined by a colorimetric assay. Cells were plated at 2 × 10⁵ cells/ml as 100 µl/well in 96-well plates and then separated by SDS-polyacrylamide gels (10% for COX-2, TNF-α, iNOS and β-actin; 7.5% for p-JNK, JNK, p-p38, p38, p65 NF-κB and inhibitory NF-κB subunit (IκBα) bands). Specific antibodies against p-JNK, JNK, p-p38, p38, p65NF-κB and inhibitory NF-κB subunit (IκBα) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Specific antibodies against COX-2 and p-IκBα were obtained from Cell Signaling Technology, Inc. (Boston, MA, U.S.A.). Antibody against iNOS was obtained from Calbiochem (San Diego, CA, U.S.A.) and the TNF-α enzyme-linked immunosorbent assay (ELISA) assay kit was obtained from Boster Biological Technology (Wuhan, Hubei, China). Reverse transcription-polymerase chain reaction (RT-PCR) reagents were purchased from TaKaRa Biotechnology (Daian, Liaoning, China). Lipopolysaccharide (LPS) (Escherichia coli, serotype 0111:B4), ERK1/2 monoclonal antibody, α-tubulin monoclonal antibody, and other reagents were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

**Cell Culture** The murine macrophage cell line RAW264.7 (American Type Culture Collection, Manassas, VA, U.S.A.) cells were cultured in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a humidified incubator containing 5% CO₂ and 95% air. The cells were plated at 2 × 10⁵ cells/ml as the initial inoculum and were used after attaining 80% confluence in culture dishes. Test compound and LPS administration was carried out under serum-free conditions.

**Cell Cytotoxicity Assay** Cellular toxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. In brief, cells were incubated with icariin derivative at concentrations of 0.8 to 80 µM. After 18 h treatment, the medium was replaced with fresh serum-free culture medium containing MTT. Formazan was dissolved in dimethyl sulfoxide (DMSO) after 4 h incubation and the optical density at 570 nm was measured and quantified.

**Measurement of TNF-α Concentrations** Cells were treated with different concentrations (0.8, 4, 20 µM) of icariin derivative for 2 h, and incubated with additional LPS (200 ng/ml) for a further 18 h. Supernatants were collected and stored at −20°C. Levels of TNF-α were determined by commercially available ELISA kits according to the manufacturer’s protocol.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction Total RNA was isolated from the cells using Trizol as suggested by the manufacturer (Invitrogen, Carlsbad, CA, U.S.A.). RNA was used for RT-PCR according to the manufacturer’s instructions. The primer sequences used for analysis of COX-2, TNF-α, iNOS and β-actin mRNA were as follows: COX-2: 5′-CCCTGCTGTGGTGGAAGCGCTTGTC-3′ (sense), 5′-TACTGAGGGTTAATGTCATCTAG-3′ (antisense). TNF-α: 5′-CGTACGCAACCACCAAGGATG-3′ (sense), 5′-CAATGACTCCAAAGTAGACC-3′ (antisense). iNOS: 5′-CTATCCGGTCGTCGGCTCGT-3′ (sense), 5′-AGCGAGTGTGGTGGATGTC-3′ (antisense). β-Actin: 5′-AGCCATGTCGAGCCATCC-3′ (sense), 5′-CTTCGACGTGGTGGTGAA-3′ (antisense). COX-2 PCR amplification was initiated with 4 min of denaturation at 94°C followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s (Mastercycler gradient; Eppendorf AG, Hamburg, Germany). β-Actin PCR amplification was initiated with 4 min of denaturation at 94°C followed by 26 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. TNF-α PCR amplification was initiated with 5 min of denaturation at 94°C followed by 27 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s. iNOS amplification was initiated with 5 min of denaturation at 94°C followed by 28 cycles at 94°C for 30 s, 52.8°C for 30 s, and 72°C for 45 s. After amplification, the samples were separated on a 2% agarose gel containing 0.5 µg/ml ethidium bromide and photographed. Digital photographs were assessed with image analysis software, and mRNA expression was evaluated as the ratio to β-actin.

**Preparation of Nuclear Fractions** For p65 NF-κB translocation analysis, protein samples of nuclear fraction were prepared. Cells were harvested, washed with ice-cold phosphate buffered saline (PBS) buffer and kept on ice. The suspension was mixed with buffer A (10 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.5, 10 mM KCl, 0.1 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N″-tetraacetic acid (EGTA), 0.1 mM ethylene diaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and protease inhibitor cocktail) and lysed by three freeze-thaw cycles. Cytosolic fractions were obtained by centrifugation at 12000 g for 20 min at 4°C. The pellets were resuspended in buffer B (20 mM HEPES, pH 7.5, 0.4 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail) and kept on ice for 40 min and followed by centrifugation at 14000 g for 20 min at 4°C. The resulting supernatant was used as soluble nuclear fractions. Equal amount proteins from nuclear fractions were used for Western blot analysis.

**Western Blot Analysis** Protein samples were prepared and then separated by SDS-polyacrylamide gels (10% for MAPks, 1×Bα, p65, 8% for iNOS). After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane. The membranes were blocked in 2% bovine serum albumin (BSA) for 1 h at room temperature, rinsed, and incubated with specific antibodies against iNOS (dilution rate of primary antibody: 1:3000), SAPK/JNK (1:1000), p-SAPK/JNK (1:1000), p38 (1:2000), p-p38 (1:1000), p-ERK1/2 (1:2000), ERK1/2 (1:2000), IκBα (1:2000), and p65 (1:2000), COX-2 (1:2000), p-1×Bα (1:1000)
overnight at 4 °C. After three washes in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) (10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and the bands were visualized by enhanced chemiluminescence and exposed to X-ray film.

**Carrageenan (CGN)-Induced Paw Edema in Mice** To further evaluate the anti-inflammatory activity of the tested compound against acute inflammation, 40 Balb/c mice (provided by Experimental Animal Center, Sun Yat-Sen University) were randomly divided into 5 groups (n=8). Two treated groups were orally administered icariin derivative (10, 30 mg/kg) for 7 consecutive days. Mice treated with vehicle or predinison (2 mg/kg) served as the control and positive control groups, respectively. Untreated mice were used as the model group. After different treatments, the mice were subcutaneously injected with 0.05 ml of carrageenan (1%) or vehicle into the right hind paw. The thickness of the paw was measured pre-injection using a vernier caliper. After the carrageenan injection, paw thickness was measured at 1, 2, 3 and 4 h. Animal studies were carried out in accordance with the Guidelines of Animal Experiments from the Ethical Committee for Animal Research of Sun Yat-sen University.

**Statistical Analysis** SPSS10.0 was used for analysis. Data are presented as the mean±S.D. from at least 3 separate experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test. A p value of less than 0.05 was regarded as a significant difference.

**RESULTS**

**Effect of Icariin Derivative on Cell Viability** Cell viability was examined to evaluate the potential cytotoxicity of icariin derivative on RAW264.7 macrophages. The results showed a low toxicity only at a concentration of 80 μM. Therefore, the concentrations used in the following experiments have no cytotoxic effect on these cells (Fig. 2).

**Icariin Derivative Inhibited Expression Levels of iNOS and COX-2 mRNA and Protein in LPS-Activated Macrophages** Semi-quantitative RT-PCR and Western blot analysis were carried out to determine whether the icariin derivative suppresses the expressions of iNOS and COX-2 mRNA and protein. Upon LPS treatment, the mRNA and protein expressions of iNOS and COX-2 were markedly aug-

![Fig. 2. Effect of Icariin Derivative on Cell Viability](image)

RAW264.7 cells were incubated with icariin derivative for 18 h (from 0.8 to 80 μM). Cell viabilities were measured using MTT assay (n=6). *p<0.05 vs. the group without icariin derivative.

Fig. 3. Icariin Derivative Inhibited TNF-α mRNA Expression and TNF-α Production in LPS-Activated RAW 264.7 Cells

(A) Cells were treated with different concentrations (0.8, 4, 20 μM) of icariin derivative for 2 h, and incubated with additional LPS (200 ng/ml) for another 18 h. TNF-α levels in culture supernatants were measured by commercial ELISA kits. The values are expressed as the mean±S.D. of three independent experiments. *p<0.05 vs. the group treated only with LPS. (B) Cells were treated with different concentrations (0.8, 4, 20 μM) of icariin derivative for 2 h, and incubated with additional LPS (200 ng/ml) for another 4 h. Total RNA of the cells was subjected to semi-quantitative RT-PCR. The RT-PCR method used is described in the Materials and Methods. One representative result of three independent experiments is shown. Values are expressed as the relative ratio (%), which was normalized to β-actin. Values are expressed as the mean±S.D. of three independent experiments. *p<0.05 vs. the group treated only with LPS.
Icariin derivative down-regulated iNOS mRNA and protein expression in a dose-dependent manner. The results showed that icariin derivative also down-regulated COX-2 mRNA and protein expression in a dose-dependent manner (Fig. 5), indicating that icariin derivative could attenuate LPS-induced iNOS and COX-2 expression at the transcriptional level.

**Effect of Icariin Derivative on Phosphorylation of MAPKs in LPS-Activated Macrophages**

Subsequent experiments were designed to elucidate the signaling cascades that induced inflammation in RAW264.7 cells in response to LPS. There is evidence that MAPKs play an important role in the control of cellular responses to stimuli. Our results revealed that phosphorylation of ERK1/2, p38MAPK, and JNK was significantly increased by treatment with LPS. Pre-treatment with icariin derivative inhibited the phosphorylation of p38 without any change in total protein expression in a dose-dependent manner (Fig. 6), whereas phosphorylation of ERK1/2 and JNK was not affected (Fig. 6).

Icariin Derivative Inhibited the Phosphorylation and Degradation of IκBα and the Nuclear Translocation of p65 Subunit of NF-κB

The heteromeric NF-κB is an inac-...
Icariin Derivative Decreased Phosphorylation and Degradation of IκBa in LPS-Activated RAW 264.7 Cells

Cells were treated with different concentrations (0.8, 4, 20 μM) of icariin derivative for 2 h, and incubated with additional LPS (200 ng/ml) for a further 15 min. Equal amounts of total protein from each group were subjected to 10% SDS-PAGE. The expression levels of p-IκBa (A) and IκBa (B) were determined by Western blot analysis. One representative result of three independent experiments is shown. Data are expressed as the mean ± S.D. of three independent experiments. * p<0.05 vs. the group treated only with LPS.

Icariin Derivative Decreased the Nuclear Translocation of p65 Subunit of NF-κB in LPS-Activated RAW 264.7 Cells

Cells were treated with different concentrations (4, 20 μM) of icariin derivative for 2 h, and incubated with additional LPS (200 ng/ml) for a further 30 min. Nuclear protein extracts were prepared as described in the Materials and Methods. Equal amounts of protein were subjected to 10% SDS-PAGE. The expression levels of NF-κB p65 were determined by Western blot analysis. One representative result of three independent experiments is shown. Data are expressed as the mean ± S.D. of three independent experiments. * p<0.05 vs. the group treated only with LPS.

Icariin Derivative Alleviated Paw Edema Induced by Carrageenan in Mice

Two treated groups were orally administered icariin derivative (10, 30 mg/kg) for 7 consecutive days. The control group received vehicle only and the positive control group received prednisone (2 mg/kg). The mice (n=8) were treated with 0.05 ml of carrageenan (1%) by subcutaneous injection into the right hind paw. The thickness of the paw was measured before injection and at intervals of 1, 2, 3 and 4 h post-injection. Each point represents the increase in paw thickness, which was calculated based on the pre-injection thickness of the paw. * p<0.05 indicates significance from the vehicle control group.

DISCUSSION

Icariin derivatives have been reported to possess multiple pharmacological activities. In MCF-7/adriamycin cells they exhibited anti-multidrug resistance ability.27) Anhydroicaritin was also found to inhibit human cytochrome P450 CYP1A2.28) Our research found that the icariin derivative 3,5-dihydroxy-4'-methoxy-6',6'-dimethyl-1,4''-5''-dihydropyrano[2'',3'',7,8]-flavone has a more potent anti-inflammatory effect compared with the others. In addition, this icariin derivative is much less cytotoxic than icariin as well as other
derivatives (data not shown). We selected it to examine its pharmacological effects on the production of inflammatory mediators in macrophages. To further elucidate the mechanism of its activity, the activations of MAPKs and the transcriptional factor NF-κB were investigated.

Our studies indicated that this icariin derivative effectively inhibits LPS-induced production of TNF-α, iNOS and COX-2 expression through suppression of NF-κB and p38 MAPK pathways. In an animal model of acute inflammation, administration of icariin derivative attenuated the paw edema induced by injection of carrageenan. The inhibitory effect of icariin derivative on inflammatory mediator expression suggests that one of the mechanisms should be responsible for its anti-inflammatory activity. It has been reported that overproduction of TNF-α play roles in many inflammation lesions, and that TNF-α elicits a number of pathological effects including septic shock, inflammation, cachexia, and cell death. It also acts as a pro-inflammatory cytokine which can induce NO synthesis. The decrease in TNF-α production by icariin derivative may suppress the further induction of inflammatory mediators such as iNOS. Aberrant expression of iNOS leads to inappropriate NO production. NO has been implicated in the pathogenesis of many disorders. Therefore, down-regulation of iNOS expression and/or its activity to suppress the level of NO generation might be useful for the treatment of inflammatory diseases. The COX system shares a number of similarities with the NOS system and they play fundamental roles in similar pathophysiological conditions such as inflammation and cancer.

Previous studies have revealed that genes of inflammation-associated enzymes and cytokines such as iNOS, COX-2 and TNF-α contain an NF-κB binding motif within their promoters. In resting cells, NF-κB is localized in the cytoplasm as a heterodimer composed of two polypeptides of 50 kDa and 65 kDa (the most common dimer), which are non-covalently associated with cytoplasmic inhibitory proteins, including an inhibitor of kappa B. Activation of NF-κB by LPS induces a cascade of events leading to the phosphorylation of 1kB and its further proteolytic degradation; the NF-κB complex migrates into the nucleus and binds DNA recognition sites in regulatory regions of target genes. In the present study we found that the phosphorylation and degradation of 1kBα and the translocation of p65 subunit of NF-κB were suppressed by icariin derivative in a concentration-dependent manner. Icariin derivative may inhibit the translocation of NF-κB into the nucleus by influencing the phosphorylation of 1kBα. Taken all together, the anti-inflammatory activity of icariin derivative may act through its interference with the NF-κB signaling pathway.

Phosphorylation of p38 MAPK, ERK1/2, and JNK induced by LPS leads to the regulation of iNOS and COX-2 expression. Studies have shown that an MAPKs inhibitor of the p38 MAPK pathways blocked nuclear NF-κB activation and the transactivation activity of p65. Carter et al. reported that p38 MAPK pathway inhibition significantly reduced binding of TATA-binding protein (TBP) to the TATA box, suggesting that p38MAPK inhibition reduced nuclear NF-κB activity by affecting the basal transcriptional complex rather than by directly affecting NF-κB itself. We observed that the p38 specific pharmacological inhibitor SB203580 inhibited the TNF-α accumulation and iNOS protein expression (data not shown), which was consistent with the findings of other researchers. However, the possible link between the activity of NF-κB and p38 MAPK activity and their contribution to inflammatory mediator regulation need to be further elucidated, because the downstream targets of the p38 pathways that affect NF-κB activity have not been identified. Our results showed that icariin derivative may inhibit proinflammatory gene expression via p38 MAPK inactivation.

A simple and reliable model of acute inflammation, carrageenan-induced paw edema in mice, was used to investigate whether icariin derivative has an in vivo anti-inflammatory effect. Paw edema, an important parameter, was measured to evaluate the potential anti-inflammatory activity of this compound. Mazzon et al. revealed that carrageenan injection caused a significant increase in TNF-α and IL-1β production, which help to propagate the extension of the local inflammatory process. In addition, iNOS and COX-2 were also induced in inflammation progress; the iNOS and COX-2 pathways appear to operate together to maintain an inflammatory response by influencing blood flow and microvascular permeability. The result obtained from this model showed that treatment with icariin derivative dose-dependently inhibited paw swelling induced by carrageenan. These findings demonstrated that icariin derivative has a potent in vivo anti-inflammatory activity via its regulation of inflammatory mediator expression.

In summary, our data showed that the icariin derivative inhibited LPS-induced inflammatory mediator expression or production through the blockage of p38 MAPK and NF-κB pathways underlying the anti-inflammatory actions of the icariin derivative. To identify the target molecules of the icariin derivative in these pathways, it is necessary to elucidate the molecular mechanism of its actions.

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