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Icariin attenuates LPS-induced acute inflammatory responses: Involvement of PI3K/Akt and NF-κB signaling pathway

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ABSTRACT

This study aimed to investigate the mechanism underlying the attenuation of LPS-induced lung inflammation by icariin in vivo and in vitro. The anti-inflammatory effects of icariin on LPS-induced acute inflammation and the molecular mechanism were investigated. Pretreatment with icariin (20 mg/kg) could attenuate acute lung inflammation by inhibiting mRNA expressions of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), metalloproteinase cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) in the lung of LPS-treated mice. In addition, icariin suppressed the secretion of TNF-α, prostaglandin E2 (PGE2) and nitric oxide (NO) as well as NF-κB p65 activation. Furthermore, decreased myeloperoxidase (MPO) activity was observed in the lung tissue and LPS-induced cytotoxicity in the RAW 264.7 macrophages cells was also markedly attenuated by icariin. Western blotting analysis and confocal microscopy showed that icariin pretreatment reduced the nucleus transportation and constant level of NF-κB p65 in the RAW 264.7 macrophage cells. However, the protective effects of icariin were reversed by a PI3K/Akt inhibitor (wortmannin). Our in vitro and in vivo results suggested that activation of the PI3K/Akt pathway and the inhibition of NF-κB were involved in the protective effects of icariin on LPS-induced acute inflammatory responses.

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1. Introduction

Lipopolysaccharide (LPS), a main component of outer membrane of Gram-negative bacteria, has been referred to be an important risk factor of acute lung injury and acute respiratory distress syndrome (ARDS) (Atabai and Matthay, 2002; Rubenfeld et al., 2005). Increasing evidence has revealed that LPS can induce the inflammatory response by activating numerous inflammatory cells and result in acute lung injury (Tumurkhuu et al., 2008; Mirzapoiazova et al., 2007).

Macrophages are important inflammatory cells implicated in the initiation of inflammatory responses, and play critical roles in the development of acute lung injury by secreting various pro-inflammatory mediators including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), metalloproteinase cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and prostaglandin E2 (PGE2). TNF-α, a classical pro-inflammatory cytokine, is involved in the early-phase of acute lung inflammation (Bhatia and Mookchala, 2004). Myeloperoxidase and its products have been important markers for evaluating oxidative damage after inflammation. Therefore, they have the potential to be specific markers for inflammation. Myeloperoxidase is an enzyme presenting in the leukocytes and a marker for leukocyte infiltration into the renal parenchyma (Donnahoo et al., 1999).

The transcription factor NF-κB plays a central role in the regulation of many genes responsible for the generation of inflammation mediators. Increased activation of NF-κB has been found in peripheral blood mononuclear cells (PBMCs), neutrophils, and alveolar macrophages in patients with acute lung injury after LPS exposure (Shao and Lin, 2008; Cloutier et al., 2007; Ndengele et al., 2005).

Recently, it has been shown that the PI3K/Akt signaling pathway plays an important role in negatively regulating LPS-induced acute inflammatory responses in vitro and in vivo (Williams et al., 2004; Guha and Mackman, 2002; Schabbauer et al., 2004a,b,c). Inhibition of the PI3K/Akt signaling pathway can enhance the activation of NF-κB, AP-1, and Egr-1 transcription factors and the expression of TNF-α, IL-6 and tissue factor by LPS in cultured human monocyctic cells (Park et al., 2009; Kim et al., 2005).

Icariin (Fig. 1), a major active component isolated from plants in the Epimedium family, has been previously confirmed to improve cardiovascular function, induce tumor cell differentiation and increase bone formation (He et al., 1995). Recently, studies have shown that icariin is a concentration-dependent chemopreventor in protecting DNA against radical-induced damage (Zhao et al., 2007) and also exerts anti-osteoporotic effects (Nian et al., 2009).
However, the potential mechanism underlying the LPS-induced inflammation remains incompletely understood. Our previous studies have shown that icariin could significantly inhibit the production of TNF-α and IL-6 in the serum of LPS-challenged mice and the supernatant of LPS-treated RAW 264.7 macrophages (Wu et al., 2009). Given that *Epimedium* has been widely used in the treatment of numerous diseases, icariin has been suggested to be beneficial for some inflammatory diseases, including acute lung inflammation. In the present study, the attenuation of LPS-induced acute inflammatory response in vitro and in vivo by icariin was explored. Results indicated the reduced secretion of inflammatory mediators and suppressed activation of NF-κB were involved in the in vitro and in vivo protective effects of icariin on the LPS-induced acute inflammatory responses.

Fig. 1. Chemical structure of icariin (C33H40O15; molecular weight = 676.67).

2. Materials and methods

2.1. Chemicals and reagents

The icariin (purity > 99.8 %) was purchased from Shanghai jiahe Bio-Pharmaceutical Company. The extract was dissolved in a culture-grade DMSO (Sigma-Aldrich, St. Louis, MO) (final concentration < 0.1%) in complete medium. LPS from *Escherichia coli* (055:B5) was obtained from Sigma-Aldrich. Reagents for cell culture including DMEM medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from GIBICO (Grand Island, NY). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl (MTT) assay system was purchased from Beyotime Institute of Biotechnology. Antibodies against phospho-Akt (S473, rabbit polyclonal antibody) and total Akt antibodies (goat polyclonal antibody) were obtained from Cell Signaling Technology; NF-κB p65 antibodies (goat polyclonal antibody) and the peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FITC-conjugated IgG (donkey anti-rabbit) was purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Wortmannin and LY294002 were purchased from Biomol (Plymouth Meeting, PA); Enzyme-linked immunosorbent assay (ELISA) kit for TNF-α, PGE2 and IL-6 was obtained from R&D Systems (Minneapolis, MN, USA); Mouse myeloperoxidase ELISA kit was purchased from Shanghai Biosis Biotechnology Co., Ltd. The NO concentrations were measured using Griess reagent systems (Promega Corporation, USA). An RNeasy™ Plus mini kit 50 was obtained from Qiagen Corporation.

2.2. Animal studies

Eleven- to twelve-week-old male C57BL/6N mice weighing 22-24 g were purchased from the Department of Laboratory Animal Science, School of Medicine, Fudan University. The animals were housed under specific pathogen-free conditions in a temperature- and humidity controlled environment and given ad libitum access to water and food. Mice were housed for 7 days for acclimation before experiments. The whole protocol was approved by the Institutional Animal Care and Use Committee of Fudan University. Mice were randomly assigned to 5 groups (n = 6-8 per group): Control group: mice were administrated (i.p.) with PBS and 1% DMSO; LPS group: mice were administrated (i.p.) with 5 mg/kg LPS; LPS plus icariin group: mice were given (i.p.) 20 mg/kg icariin 2 h before LPS treatment; LPS + icariin + wortmannin group: icariin and wortmannin (0.3 mg/kg) was given (i.p.) simultaneously (Schabbauer et al., 2004b). Four hours after PBS (control) or LPS (all other groups) treatment, the animals were killed, and blood and tissues were obtained for analysis.

Lungs were fixed in 10% paraformaldehyde, embedded in paraffin, cut into 4 μm sections and stained with hematoxylin and eosin (HE). For the acute airway inflammation, the grading system was as follows (Singh et al., 2005): grade 0, no or occasional cells; grade 1: a few loosely arranged cells; grade 2, many cells in the peripheral parts of the perivascular space; grade 3, numerous cells in the perivascular space.

2.3. Cell culture and treatment

RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection and maintained in DMEM containing 10% FBS, 100 kU/L penicillin, and 100 mg/L streptomycin. In all experiments, the cells were subject to no more than 20 passages. To examine the effects of icariin and LPS on the cell viability, MTT assay was performed at various time points after LPS treatment according to the manufacturer's instructions.

To assess the effects of icariin on the Akt phosphorylation, cells were serum-starved for 16 h, washed once, and then incubated without or with 10 μM LY294002 for 60 min before incubation with icariin (0.01–2 μM) in serum-free medium for different times.

2.4. Lung myeloperoxidase assay

Inflammatory cell sequestration in pancreas and lungs was quantified by measuring tissue myeloperoxidase activity (Bhatia et al., 2005). The lungs (n = 6 per group) were weighed and homogenized in ice-cold 50 mM potassium phosphate buffer (pH 7.4) followed by centrifugation at 13,000 rpm for 10 min at 4 °C. The pellet was re-suspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% wt/vol hexadecyltrimethylammonium bromide (Sigma). The absorbance was measured at 450 nm and normalized by the amount of DNA in the tissues. The results were expressed as enzyme activity.

2.5. Measurement of TNF-α, PGE2 and NO levels in serum

After treatment, serum was separated by centrifuging at 600 g for 15 min at 4 °C. Aliquot serum was stored at −80 °C for use. The levels of TNF-α and PGE2 in the serum were analyzed using ELISA kits. The concentration of NO in serum was determined as that of nitrite, a major stable product of NO, using the Griess reagent systems (Dynatech MR-7000; Dynatech Laboratories) (Jung et al., 2009). All assays were performed as described by the manufacturer's instructions.

2.6. RNA extraction and real-time polymerase chain reaction (real-time RT-PCR)

After treatment, the RNeasy™ Plus mini kit was used to isolate the total RNA from the lung tissues, and the quality of RNA was subsequently evaluated by measuring the ratio of the absorbance at 260/280 nm. For reverse transcription, the SYBR Green 2-step qRT-PCR kit (DyNAmo™, Finnzymes, Finland) was used. For PCR amplification, the following mouse-specific sense and antisense primers were used: TNF-α, 5′-CCGACGCTGGAGGAGAAGG-3′ (forward) and 5′-TCCAGTACCCAGACAGA-3′ (reverse); IL-6, 5′-TGGTCTCAAGAGGAGTTG-3′ (forward) and 5′-ACCCCTCAAGAGGAGTTG-3′ (reverse); iNOS, 5′-TGGACTCACAGAGAGGACTGC-3′ (forward) and 5′-TCTGACACAGGAGGAGTTGAC-3′ (reverse); COX-2, 5′-
GGAGAGACTAAGATAG TGATC-3’ (forward) and 5’-ATGGCTAGTAGGCTTTACAGCTC-3’ (reverse); GAPDH, 5’-GGGGAGCCAAAAGGTCATC-3’ (forward) and 5’-GACGCCTGCTTCACCACCTTCTTG-3’ (reverse). Real-time quantitative PCR was carried out in a 48-well plate using an Opticon MJ Research instrument (Bio-rad Inc., USA) and an optimized standard SYBR Green 2-step qRT-PCR protocol.

2.7. Western blot analysis

RAW 264.7 cells were washed three times with ice-cold PBS containing 1 mM Na3VO4 and 5 mM EDTA after treatment, and then re-suspended in 0.2 ml of lysis buffer. For fresh tissues, tissues were put into Microtainer tubes containing heparin and then centrifuged at 1500 g for 10 min at 4 °C. The pellets were washed twice with ice-cold PBS containing 1 mM Na3VO4 and 5 mM EDTA (Ethylene Diamine Tetraacetic Acid), and then re-suspended in 0.5 ml of lysis buffer. After incubation of the cell lysates on the ice for 20 min and centrifugation at 14,000 g for 15 min at 4 °C, the supernatant was obtained and stored at −80 °C for analysis.

After preparation for cytoplasmic and nuclear extracts, proteins were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were then blocked with 10% non-fat milk in Tris-buffered saline containing 0.2% Tween 20 (TBS-T) at room temperature for 3 h and incubated with the following primary antibodies in TBS-T: NF-κB p65 (1:500), phospho-Akt (1:1,000), Akt (1:1000) and β-actin (1:500) overnight at 4 °C, with continuous shaking. The membranes were then washed with TBS-T and subsequently incubated with secondary antibodies conjugated to horseradish peroxidase (1:2000) for 1 h. The bands were visualized using luminol reagent and X-ray film. Band intensities were quantified using UN-SCAN-IT gel analysis software (version 6). The fold increase in the level of protein expression was calculated by comparing it with that of normal controls.

2.8. Confocal microscopy

The nuclear localization NF-κB p65 was examined by immunofluorescence assay indirectly. RAW 264.7 cells were maintained on the glass coverslips in 24-well plates for 24 h. After stimulated with LPS (1 μg/ml) and/or icariin (2 μM), the cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, and blocked with 1.5% normal donkey serum (Sigma). Polyclonal antibodies to NF-κB p65 were applied for 1 h, followed by 1 h of incubation with FITC-conjugated donkey anti-rabbit IgG. After washing with PBS, the coverslips were mounted in Fluoromount-G™, and the fluorescence was visualized using a Zeiss LSM 510 Meta microscope.

2.9. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously (Nicholas et al., 2007). In brief, 30 min before LPS stimulation, RAW 264.7 cells were pre-incubated with indicated
concentrations at 37 °C. Then, cells were stimulated with 1 μg/ml LPS for the indicated times, and washed twice with ice-cold PBS. Then nuclear extracts were prepared and incubated for 20 min at room temperature with a gel shift binding buffer [5% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl, pH 7.5, and 50 g/ml poly(dl-dC)] and 32P-labeled oligonucleotide. The DNA–protein complex was separated by 4% native polyacrylamide gels. The gel was transferred to the Whatman 3 MM paper (Clifton, NJ), which was dried, and exposed to X-ray film.

2.10. Statistical analysis

Data were from three independent experiments and expressed as mean ± S.E.M. Statistical analyses were performed by the Student’s t test for paired data and the one-way Analysis of Variance (ANOVA) for differences between treatment groups. All analyses were undertaken using statistic software SPSS 11.0. A value of P<0.05 was considered statistically significant.

3. Results

3.1. Icariin suppressed LPS-induced recruitment of lung inflammatory cells in mice

To examine whether icariin attenuated LPS-induced acute lung injury, C57BL/6N mice were treated with icariin plus LPS. Four hours later, LPS-induced infiltration of inflammatory cells was evident in the airway and the perivascular space as compared to controls. Furthermore, icariin pretreatment markedly diminished the infiltration of inflammatory cells when compared to the mice with LPS treatment.

A

![Graph: Effects of icariin treatment on the lung myeloperoxidase. The level of myeloperoxidase in the 5 groups [PBS (1×), DMSO (1%), LPS (50 mg/kg), LPS + icariin (20 mg/kg), LPS + icariin + wortmannin (0.3 mg/kg)]. *P<0.05 vs LPS group (n = 6).]

B

![Graph: mRNA expression of inflammatory mediators in LPS-induced acute lung inflammation. The level of TNF-α, IL-6, iNOS, and COX-2 in the 5 groups [PBS, DMSO, LPS, LPS + icariin, LPS + icariin + wortmannin]. *P<0.05 vs LPS group. #P<0.05 vs LPS group.]

C

![Graph: Serum levels of NO in the 5 groups [PBS, DMSO, LPS, LPS + icariin, LPS + icariin + wortmannin]. *P<0.05 vs LPS group.]

D

![Graph: Serum levels of PGE2 in the 5 groups [PBS, DMSO, LPS, LPS + icariin, LPS + icariin + wortmannin]. *P<0.05 vs LPS group.]

Fig. 3. Effects of icariin treatment on the lung myeloperoxidase. The level of myeloperoxidase in the 5 groups [PBS (1×), DMSO (1%), LPS (50 mg/kg), LPS + icariin (20 mg/kg), LPS + icariin + wortmannin (0.3 mg/kg)]. *P<0.05 vs LPS group (n = 6).

Fig. 4. Icariin reduced the protein and mRNA expression of inflammatory molecules in LPS-induced acute lung inflammation. Mice were treated with PBS, vehicle, LPS, icariin and wortmannin as described in Materials and methods. At 4 h after treatment, the animals were killed, lung tissues and blood were collected, mRNA expression of inflammatory mediators was determined by real-time quantitative RT-PCR (panel A) and serum levels of TNF-α, and PGE2 were measured by ELISA (panels B and C). The concentrations of NO in the serum were determined as nitrite, using the Griess reagent system (Fig. 4D). Data were presented as means ± SE from three independent experiments. * P<0.05 vs LPS group. # P<0.05 vs LPS group.
mice. The mRNA expression of TNF-α, IL-6, iNOS and COX-2 by 22.9%, 39.4% and 43.2% (P<0.05 vs LPS group), respectively. Although the LPS-induced expression of IL-6 was also reduced, no significant difference was found between the icariin + LPS group and LPS group (Fig. 4A). In contrast, there were no statistically significant differences in the induction of TNF-α, NO and PGE₂ between the LPS group and LPS plus icariin group (Fig. 4B, C, D).

Furthermore, pretreatment with wortmannin before icariin and LPS treatment diminished the protective effects of icariin. Therefore, these results indicated that the PI3K/Akt pathway played a critical role in negatively regulating the in vivo production of inflammatory cytokines and the protective effect of icariin might be mediated by activation of the PI3K/Akt pathway.

3.4. Effects of icariin on the viability of RAW 264.7 macrophages cells

The RAW 264.7 cells were grown in serum-free medium and treated with LPS (1 μg/ml) for 4, 8, 12 and 24 h. The cell viability was assessed by MTT assay. (A) showed that cell viability was greatly decreased in a time dependent manner; (B) indicated, after icariin treatment (0.01-2 μM) for 24 h, LPS-induced cytotoxicity was markedly inhibited. The protective effects of icariin could be achieved when the concentration of icariin was 0.5 μM or greater (P<0.05 and 0.001 vs LPS group). Different concentrations (0.01–2 μM) of icariin did not affect cell viability in RAW 264.7 mouse macrophage cells (data not shown) (Fig. 5). No significant difference in the cell viability was observed between icariin treatment groups in which icariin of different concentrations was applied.

3.5. Icariin inhibited the LPS-induced NF-κB activation in vitro and in vivo

We also investigated whether icariin could interfere with the translocation of p65 subunit of NF-κB from the cytosol to the nucleus in RAW 264.7 macrophages. Confocal microscopy revealed that, in unstimulated cells, NF-κB p65 was mostly presented in the cytoplasm. After LPS treatment, most of the intracellular p65 had translocated from the cytoplasm to the nucleus, as shown by strong NF-κB p65 staining in the nucleus (Fig. 6A). The level of p65 in the nucleus was significantly reduced by pretreatment with icariin (2 μM) and PDTC (10 μM) in immunofluorescence assay. All immunofluorescent staining was performed simultaneously.

The LPS-induced NF-κB activity was inhibited by 25.8% (P<0.05 vs LPS group) by icariin pretreatment (20 mg/kg) in mice. However, the suppressed NF-κB activity by icariin treatment was reversed by wortmannin (0.3 mg/kg body weight) treatment (Fig. 6B). It has been known that the PI3K/Akt pathway is implicated in the negative regulation of NF-κB activation and expression inflammatory cytokines (Schulze-Luehrmann and Ghosh, 2006; Holgate and Polosa, 2008).

These findings indicated that the activation of the PI3K/Akt pathway by icariin partially inhibited the NF-κB activation and the inhibitory effects of icariin on the LPS-induced up-regulation of TNF-α, iNOS and COX-2 were conferred by inhibiting the NF-κB activation.

We further investigated whether icariin could prevent the translocation of p65 subunit of NF-κB from the cytosol to the nucleus. Results showed that icariin pretreatment attenuated the p65 level in the nucleus and subsequently increased the cytosolic p65 level as compared to the LPS group (Fig. 6C). The level of p65 in the nucleus was significantly reduced by 5 μM icariin which was demonstrated by Western blot analysis.

EMSA was performed to determine whether the anti-inflammatory effects of icariin involved the NF-κB activity. In the EMSA assay, nuclear extracts were obtained from RAW 264.7 cells after stimulation with LPS for 1 h in the presence or absence of icariin. LPS (1 μg/ml)
treatment significantly increased the NF-κB-DNA binding, which was dramatically abolished by icariin treatment (2 μM) (Fig. 6D).

3.6. Icariin activated the PI3K/Akt signaling pathway in RAW264.7 cells

The PI3K/Akt signaling pathway has been recently shown to negatively regulate the LPS-induced acute inflammation responses in vitro and in vivo (Schabbauer et al., 2004c). With the same model, we investigated whether icariin could activate the PI3K/Akt pathway and the LPS-induced Akt phosphorylation in RAW 264.7 cells. Incubation of RAW 264.7 cells with 0.01–2 μM icariin for 4 h could induce the phosphorylation of serine residue 473 of Akt in a concentration-dependent manner. For example, the level of phosphorylated Akt was markedly increased in cells treated with 0.01–2 μM icariin for 4 h when compared to untreated controls (P<0.05) (Fig. 7A). Peak levels of Akt phosphorylation were achieved after 4 and 1 h of incubation with 1 μM and 5 μM icariin, respectively. Pre-incubation of RAW 264.7 cells with the PI3K inhibitor LY294002 (10 μM) abolished the icariin-induced phosphorylation of Akt which was independent of the icariin concentration (Fig. 7B). These findings strongly suggest that icariin could induce Akt phosphorylation by activating the PI3K pathway.

4. Discussion

In the present study, our results demonstrated icariin played an important role in preventing against LPS-induced acute inflammatory responses in mice and RAW 264.7 macrophages. Pretreatment with icariin attenuated lung injury by inhibiting the production of inflammatory mediators and NF-κB activation, which was found to be associated with the activation of the PI3K/Akt signaling pathway. LPS is a glycolipid presenting in the outer membrane of Gram-negative bacteria and composed of a polar lipid headgroup (lipid A) and a chain of repeating disaccharides (Raetz et al., 1991). LPS binding a specific LPS binding protein (LBP) generates a LPS/LBP complex which then activates the CD14/TLR4 receptor on the monocytes, macrophages, etc, and triggers the transduction of inflammatory signal resulting in the regulation of the inflammatory cytokine.
expression (Yang et al., 1998; Tapping et al., 2000; Wright et al., 1990). Lung is the most frequently involved organ, and LPS induced acute lung injury is the main cause of death.

ARDS is a severe complication of stress situations such as trauma, burns and sepsis, which may lead to non-cardiogenic respiratory failure. The use of bacterial LPS has a number of advantages as a method to model the effects of Gram-negative bacteria in animals and humans (Rosenthal et al., 1998; Windsor et al., 1993; Flick, 1986). Therefore, acute lung injury was reproduced in the present study and lung pathology showed the infiltration of inflammatory cells into the airway and the perivascular space.

TNF-α is secreted by a variety of cells, including macrophages, lymphocytes, mast cells and epidermal cells, however, it mainly comes from macrophages (Gong et al., 2008). By activating macrophages and natural killer cells, numerous biologically active substances are released as important toxic substances, which participate in anti-infection, shock, fever, organ failure and other pathophysiological process (Mehta, 2005).

Our previous studies have shown that icariin could significantly inhibit the production of TNF-α and IL-6 in the serum of LPS-challenged mice and the supernant of LPS-treated RAW 264.7 macrophages. Furthermore, we herein provided evidence that icariin exhibited potent anti-inflammatory effects in vitro and in vivo by attenuating LPS-induced lung injury and the mRNA expression of TNF-α, IL-6, iNOS and COX-2 in the lung of LPS-treated mice. The present results were consistent with those in our previous observations, which also showed intraperitoneal administration of 20 mg/kg icariin exerted anti-inflammatory effects in mice. In addition, decreased myeloperoxidase activity was also observed in the tissue, suggesting decreased neutrophil infiltration into the lung although no or a small amount of expression of myeloperoxidase is found in the normal lungs. Significant difference in the expression of myeloperoxidase was noted between the icariin + LPS group and control group, indicating icariin pretreatment could inhibit the expression of myeloperoxidase in mice and reduce the inflammatory cascade.

NF-κB activation has been known to regulate various cellular responses, including apoptosis, and is required for the inductive expression of inflammation related and tissue-repair related genes (Ghosh and Hayden, 2008). It is well documented that NF-κB plays a critical role in the LPS-induced transcriptional regulation of most inflammatory genes contributing to the development of anaphylactic disease, septic shock, multiple organ failure, and even death (Liang et al., 2004; Karin and Greten, 2005; Li and Verma, 2002). Phosphorylation of p65 at the serine 536 in the transactivation domain enhances the transcriptional activity of NF-κB (Viatour et al., 2005; Takeshima et al., 2009). Protein kinases are also required for the optimal NF-κB activation by targeting functional domains of NF-κB protein itself. Phosphorylation of the p65 subunit plays a pivotal role in determining both the strength and duration of the NF-κB-mediated transcriptional response. In our experiment, after LPS treatment, most of the intracellular p65 had translocated from the cytoplasm to the nucleus, demonstrated by strong NF-κB p65 staining in the nucleus. The level of p65 in the nucleus was significantly reduced by pretreatment with icariin (2 μM) and PDTC (10 μM). These results suggested icariin could inhibit LPS-induced acute inflammatory responses which were mediated by the NF-κB signaling pathway.

The PI3K/Akt pathway has also been shown to control a variety of cellular processes, including cell survival and proliferation (Cantley, 2002). The PI3K/Akt pathway involves a conserved family of signal transduction enzymes that are implicated in regulating cellular proliferation and survival. A growing body of evidence suggests that the PI3K/Akt pathway plays an important role as a negative feedback regulator of excessive innate immune and Toll-like receptor-mediated pro-inflammatory responses (Kim et al., 2005).

Recently, numerous studies have shown that the PI3K/Akt signaling pathway plays an important role in negatively regulating LPS-induced acute inflammatory responses in vitro and in vivo (Williams et al., 2004; Guha and Mackman, 2002; Schabbauer et al., 2004c). However, the role of PI3K/Akt signaling cascades in the regulation of NF-κB transactivation remains controversial (Takeshima et al., 2009). The present study agrees with previous investigations which demonstrate that PI3K/Akt activation promotes the p65 activation (Arbibe et al., 2000). However, other studies showed the
inhibition of the PI3K/Akt pathway augmented the p65 activation (Guha and Mackman, 2002).

In the present study, when the animals were treated with wortmannin before icarrin and LPS treatment, the protective effects of icarrin were abolished. These findings indicate the PI3K/Akt pathway plays a crucial role in negatively regulating the in vivo production of these inflammatory cytokines, and the protective effects of icarrin are mediated by the activation of PI3K/Akt pathway. We also explored how the LPS-stimulated PI3K activation led to the inhibition of NF-κB activation, and identified a pathway initiated by the PI3K activation that was independent of NF-κB DNA binding. Pretreatment with icarrin (20 mg/kg) inhibited the LPS-induced NF-κB activity by 25.8% in mice. However, when the mice were treated with wortmannin (0.3 mg/kg body weight) before icarrin and LPS treatment, the inhibitory effects of icarrin on the serum level and the mRNA expression of TNF-α, iNOS, PG E2 and COX-2 in the lungs of LPS-treated mice were reversed.

Furthermore, pretreatment of RAW 264.7 macrophages with wortmannin resulted in the marked enhancement of the LPS-induced p65 phosphorylation and the ability of LPS to activate NF-κB dependent transcription. Therefore, these results established a clear role of PI3K and its downstream effector Akt in modulating the transactivation of p65. Further studies are required to define the exact signaling cascade involved in the LPS-induced p65 phosphorylation and NF-κB activation.

In conclusion, wortmannin, a pharmacological inhibitor of PI3K, was used to test the hypothesis that icarrin can decrease the production of pro-inflammatory cytokines and NF-κB activation in the LPS-induced acute inflammatory responses in vitro and in vivo, in which the PI3K/Akt pathway plays an important role. Our results demonstrated that the PI3K/Akt pathway negatively regulated the NF-κB signaling in the LPS-stimulated RAW 264.7 macrophages, and icarrin exerted anti-inflammatory effects through the PI3K/Akt pathway.

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