Icaritin exhibits anti-inflammatory effects in the mouse peritoneal macrophages and peritonitis model

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A B S T R A C T

Icaritin, an intestinal metabolite of prenylflavonoids from Herba Epimedii, has been known to regulate many cellular processes. The purpose of this study was to investigate the protective effects of icaritin on inflammation in lipopolysaccharide (LPS) stimulated mouse peritoneal macrophages in vitro and zymosan induced peritonitis model in vivo. The release of Nitric oxide (NO) was measured by a Griess reagent system. The phagocytosis, the expression of CD69, the production of inflammatory cytokines and the leukocytes numbers were determined by flow cytometry. The Ca²⁺ influx was recorded by confocal microscopy. The phosphorylation of p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) was determined by Western blot. The results showed that icaritin significantly inhibited the NO, IL-6, IL-10 TNF-α, and MCP-1 production both in vitro and in vivo. Icaritin efficiently diminished the uptake of nonopsonized pHrodo−Escherichia coli bacteria on the LPS-stimulated macrophages. In addition, icaritin significantly inhibited the expression of CD69 on CD11b+ macrophages. Icaritin pretreatment significantly inhibited the elevation of intracellular Ca²⁺ induced by LPS. Furthermore, icaritin markedly decreased phospho-p38 and JNK protein expression in LPS-stimulated mouse peritoneal macrophages. In vivo study, it was also observed that icaritin prolonged survival of peritonitis mice, and inhibited massive leukocyte influx into the peritoneal cavity. These results suggest that icaritin possesses significant anti-inflammatory effects that may be mediated through the regulation of inflammatory cytokines and phosphorylation of p38 and JNK.

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1. Introduction

Inflammation is a complex pathophysiological process, which is mediated by the host’s microcirculatory system to protect against pathological states such as irritation and infection caused by chemicals, microbial pathogens. However, dysregulated or unbalanced inflammation may cause progressive tissue damage and has been evolved in the development of many chronic diseases such as cancer [1]. Inflammation is characterized by recruitment of various immune cells (e.g. macrophages) to the inflammatory sites and the expression of proinflammatory mediators [2]. Lipopolysaccharide (LPS), the production of Gram-negative bacteria, is recognized by macrophages and subsequently activates macrophages. Therefore, LPS-induced macrophages is often used to examine the release of a wide range of inflammatory mediators, including nitric oxide (NO), IL-6, IL-10, IL-12p70, IFN-γ, MCP-1, TNF-α in vitro [3]. Moreover, mitogen-activated protein kinase (MAPK) is one of the important regulators of inflammatory mediators, such as NO and proinflammatory cytokines. In addition, recent studies have found that MAPK pathway is related to the production of mediators in LPS-induced mouse peritoneal macrophages [4].

Herba Epimedii, the dried aerial parts of the epimedium plants, has been used as a tonic effect in oriental countries, especially China [5]. Icarin is recognized as the major active ingredient of the herb [6,7], which can be hydrolyzed to its metabolites, including icaritin (ICA, Fig. 1) [8,9]. As a highly interesting natural flavonoid compound for drug development, icaritin has a broad spectrum of established pharmacological functions, including inhibition of human endometrial cancer cells [10], breast cancer cells [11], human prostate carcinoma PC-3 cells [12], and chronic myeloid leukemia [13]. Including anti-cancer effect, recent studies have reported that icaritin reduces incidence of steroid-associated osteonecrosis with inhibition of both thrombosis and lipid-deposition in a dose-dependent manner [14]. In addition, icaritin also shows an immunosuppressive effect on T cells by interfering T cells activation [15]. However, the effect of icaritin on inflammation in the macrophages remains to be elucidated.

In the current report, we examined the impact of icaritin treatment in vitro using LPS-activated murine peritoneal macrophages.
Zymosan-induced peritonitis, as a well-characterized model for studies on acute peritoneal inflammation [16], was applied to evaluate the effect of icaritin administration in vivo.

2. Materials and methods

2.1. Materials

Icaritin (ICA, HPLC purity > 99.5%) was supplied by Shenogen Pharma Group (Beijing, China). Lipopolysaccharides (LPS), ethylene glycol tetra-acetic acid (EGTA) and zymosan were obtained from Sigma (USA). 1.2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) was purchased from Enzo Life Sciences International (USA). RPMI 1640 medium, fetal bovine serum (FBS), glutamine, β-mercaptoethanol, penicillin and streptomycin were purchased from Gibco Lab (Rockville, MD). Anti-mouse CD11b-PE, anti-mouse CD69-FITC, Absolute Counting Tubes and Cytometric Bead Array (CBA) Mouse Inflammation Kit were obtained from BD-PharMingen (USA). Cell Counting Kit-8 was purchased from DOJINDO (JAPAN). Griess Kit was obtained from Promega (USA). Fluo-4/acetoxymethyl ester (AM), Pluronic F-127, pHrodo™ E. coli BioParticles Kit were obtained from Invitrogen (USA). JNK Mouse mAb, Phospho-JNK Mouse mAb, Erk1/2 Mouse mAb, Phospho-Erk1/2 Mouse mAb, p38 Mouse mAb and Phospho-p38 Mouse mAb were purchased from Cell Signaling (USA).

2.2. Animals

Female BALB/c mice at the age of 6–8 weeks were purchased from the Experimental Animal Center of Southern Medical University (Guangzhou, China). The animal experiments were approved by the Ethics Committee for Experimental Animals at Jinan University and were performed according to the national guidelines for animal welfare.

2.3. Cell isolation and culture

Peritoneal macrophages were isolated as described previously with minor modifications [17]. Briefly, BALB/c mice were sacrificed by cervical dislocation. About 10 ml of cold RPMI 1640 medium (containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine and 50 μM β-mercaptoethanol) was injected into the abdominal. The abdomen was gently massaged for 2 min. The medium was drawn back and the peritoneal fluid was collected. The peritoneal cells were re-suspended in the complete RPMI 1640 medium containing 10% FBS. Cells at 1 × 10^6/ml in RPMI 1640 medium were seeded on a 24 well plate with 500 μl in each well, and the plate was incubated overnight at 37 °C and 5% CO₂ tension. The cells were then washed two times with medium to remove non-adherent cells. The adherent cells (97% macrophages) were incubated at 37 °C and 5% CO₂ tension in medium containing 10% FBS for growth.

2.4. Cytotoxicity

Peritoneal macrophages were seeded on a 24-well plate and were pretreated with different concentrations of ICA (2.5, 5 and 10 μM) for 4 h, and then co-treated with 5 μg/ml of LPS at 37 °C. After being cultured for 48 h, cells were added with 40 μl CCK-8 and incubated 4 h. The absorbance was then measured at 450 nm using a microplate reader (Bio-Rad, USA).

2.5. NO assay

The levels of NO in the medium of macrophages were measured using a Griess reagent system kit according to the manufacturer’s instructions. Briefly, 50 μl of culture supernatants was gently mixed with an equal volume of sulfanilamide solution and incubated in the dark at room temperature (RT) for 8 min. After the incubation, 50 μl of naphthylethlenediamine dihydrochloride was added to the reaction and incubated in the dark at RT for another 8 min. The absorbance at 540 nm was measured in a microplate reader. Nitrite concentration, an indicator of NO production, was calculated from a NaNO₂ standard curve.

2.6. Phagocytosis assay

The flow cytometry phagocytosis assay was performed using a pHrodo™ E. coli BioParticles Kit according to the manufacturer’s instructions. Briefly, peritoneal macrophages were seeded on a 24-well plate and were exposed at various concentrations of ICA (2.5–10 μM). After being stimulated by LPS (5 μg/ml) for 12 h, cells were added with 5 μL E. coli BioParticles labeled with pHrodo™ and incubated in the dark for 4 h. The cells were harvested by a cell scraper and washed two times. The flow cytometry analysis was then performed using a FACSArria flow cytometer (BD, USA).

2.7. Membrane staining

Peritoneal macrophages were seeded on a 24-well plate and were exposed at various concentrations of ICA (2.5–10 μM). After being stimulated by LPS (5 μg/ml) for 24 h, cells were harvested by a cell scraper and washed two times. The cells were incubated with anti-mouse CD11b-PE mAb and CD69-FITC mAb in the dark for 30 min, followed by washing two times. The flow cytometry analysis was then performed using a FACSArria flow cytometer (BD, USA).

2.8. Intracellular Ca²⁺ kinetics

Intracellular Ca²⁺ kinetics was performed as described previously with minor modifications [18]. Briefly, peritoneal macrophages were seeded on a petri dish (MatTek, USA). The Ca²⁺ indicator Fluor-4 AM was gently mixed with an equal volume of pluronic F-127. The final concentration of fluorescent calcium dye is 2.5 μM. Cells were loaded with the mixture and incubated in the dark at 37 °C for 30 min. After the incubation, cells were washed by Locke’s buffer (145 mM NaCl, 5 mM KCl, 2.6 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES-Na, and 5.6 mM glucose adjusted to pH 7.4 with HCl). The cells were suspended with Locke’s buffer, followed by the addition of EGTA and BAPTA-AM. The cells were pretreated with 5 μM ICA for 10 min and then stimulated by 10 μg/ml LPS. After 1200 s of recording, 2 mM Ca²⁺ were administered. The imaging of time series was performed by LSM 510META confocal microscope (Zeiss, Germany). The regions of interest (ROIs) was analyzed by the Zeiss AIM Image Examiner software.
2.9. Inflammatory cytokine assay

Cytokine secretions of peritoneal macrophages were analyzed by mouse inflammation CBA Kit following the manufacturer’s instruction. This kit allows detecting six cytokines (IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70), simultaneously. Macrophages were seeded on a 24-well plate and were pretreated with 5 μM ICA. After being stimulated by LPS (5 μg/ml) for 24 h, 50 μl of culture supernatants was gently mixed with an equal volume of the mixed captured beads. All assay tubes were added 50 μl of the PE detection reagent and incubated in the dark at room temperature for 2 h. After the incubation, each assay tube was added 1 ml of Wash Buffer and centrifuged at 200 g for 5 min, followed by discarding the supernatant. The bead pellet was resuspended with 300 μl of Wash Buffer for the flow cytometry analysis.

2.10. Western blot

Immunoblotting was performed as described previously with minor modifications [19]. Briefly, peritoneal macrophages were seeded on a 24-well plate and were exposed at various concentrations of ICA (2.5–10 μM). After being stimulated by LPS (5 μg/ml) for 2 h, cells were harvested by a cell scraper and washed twice, followed by the addition of 100 μl of RIPA lyse solution (Biocolors, China) supplemented with 1 μl of PMSF on the ice for 1 h. The lysates were centrifuged at 12,000 rpm for 20 min at 4 °C. Supernatant was collected and protein concentration in samples was estimated by the protein assay reagent (Bio-Rad, USA) following the manufacturer’s instructions. The samples containing 20 μg of protein were separated on 10% sodium dodecyl sulfate-poly-acrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated for 1 h with 3% BSA in TBS buffer (0.1 M Tris-HCl, pH 7.4, 0.9% NaCl) to block non-specific binding, followed by incubation with primary antibody (JNK mAb, Phospho-JNK mAb, Erk1/2 mAb, Phospho-Erk1/2 mAb, p38 mAb, Phospho-p38 mAb). Subsequently, the membranes were incubated with secondary antibody streptavidin-horseradish peroxidase-conjugated IgG. Specific bands were visualized by ECL according to the manufacturer’s instruction. Densitometry was performed using FluorChem 8000 image system (Alpha).

2.11. Zymosan-induced peritonitis

Mice were randomly allocated into the following groups:
1. ZYM group. Mice were treated intraperitoneally (i.p.) with zymosan (50 mg/kg, suspended in normal saline) and with the vehicle for ICA (0.1% DMSO, v/v, i.p.) at 1 and 6 h after zymosan administration (N = 10).
2. ICA group. Identical to the ZYM group but were administered ICA (4 mg/kg, i.p.) instead of DMSO (N = 10).
3. SHAM group. Identical to the ICA group but were administered normal saline (i.p.) instead of zymosan (N = 10).

All mice were monitored for systemic toxicity and mortality for 13 days after zymosan or saline administration. In another set of experiments, animals (n = 10 for each group) were randomly divided as described above, but ICA or vehicle was pretreated. All mice were sacrificed by cervical dislocation. At 6 h or 24 h after zymosan or saline administration, the peritoneal exudates were collected for the assay of NO, inflammatory cytokines as described above, and leukocytes numbers.

2.12. Leukocytes numbers

Leukocytes from the peritoneal exudates were counted using the Absolute Counting Tubes following the manufacturer’s instruction. Briefly, 20 μl of normal saline was dispensed into a TruCount tube, just above the stainless steel retainer, avoiding touching the pellet. After the pellet was dissolved, 500 μl of the peritoneal exudates was added into the tube. The tube was then capped and vortexed gently to mix, followed by the analysis of flow cytometry.

2.13. Statistical analysis

Data were expressed as mean ± SD. Statistical significance was analyzed by one-way ANOVA with Student’s test. Significant difference was considered when P < 0.05.

3. Results

3.1. Effect of icaritin on cell viability in LPS-induced peritoneal macrophages

First, the effect of icaritin on the cell viability in LPS-induced peritoneal macrophages was assessed using CCK-8 assay. We found that treatment with ICA (2.5, 5 and 10 μM) for 48 h did not cause any significant peritoneal macrophages viability change vs control group with or without LPS (Fig. 2), indicating that ICA was non-cytotoxic to peritoneal macrophages within experimental concentration range.

![Fig. 2. Effect of ICA on the cell viability of peritoneal macrophages.](image_url)
3.2. Effect of icaritin on NO release in LPS-induced peritoneal macrophages

NO is an important cellular signaling molecule, which is generated by macrophages. Then, the release of NO in LPS-induced macrophages was examined. LPS significantly induced NO release in macrophages. However, the application of ICA (2.5, 5 and 10 \( \mu \)M) inhibited the LPS-induced NO production in a dose-dependent manner (Fig. 3). However, no significant change was observed on the macrophages treated with ICA alone, compared to the untreated cells.

3.3. Effect of icaritin on the phagocytic capacity in LPS-induced peritoneal macrophages

Using pHrodo-labeled \( E. \) coli particles is a quick and reliable method to evaluate the phagocytic activity of macrophages. We found that the phagocytosis of cells was significantly increased after LPS activation. However, ICA considerably inhibited the engulfment of particles by the activated macrophages within experimental concentration range (Fig. 4). However, compared to the untreated cells, no significant change was observed on the macrophages treated with ICA alone.

3.4. Effect of icaritin on the CD69 expression in LPS-induced peritoneal macrophages

In mouse, the CD11b antigen is highly expressed on macrophages. Moreover, CD69 is one of the earliest cell surface molecules expressed by activated macrophages. Therefore, two-color flow cytometry was performed to assess the activation marker on LPS-stimulated macrophages, and compensation setting and gates were established on control. We observed that ICA significantly inhibited the expression of CD69 on the CD11b+ cells within experimental concentration range (Fig. 5F).

3.5. Effect of icaritin on the \( \text{Ca}^{2+} \) influx in LPS-induced peritoneal macrophages

To block the elevation of calcium, macrophages were preloaded with the intracellular calcium chelator BAPTA-AM (50 \( \mu \)M), and extracellular calcium was depleted by the addition of EGTA (50 \( \mu \)M) to the medium. As shown in Fig. 6, the LPS-induced calcium elevation was nearly abolished since the intra- and extracellular calcium were both chelated by BAPTA-AM and EGTA. The response to the stimuli...
was efficiently restored by adding Ca\(^{2+}\) back to the suspensions. However, we found that pretreatment of ICA at 5 \(\mu\)M significantly inhibited the Ca\(^{2+}\) influx induced by the stimuli of LPS.

3.6. Effect of icaritin on the inflammatory cytokines production in LPS-induced peritoneal macrophages

To further characterize the effect of ICA on the release of inflammatory cytokines in peritoneal macrophages, we analyzed cytokines secretion by LPS-induced macrophages that have been cultured with or without ICA. Our data showed that pretreatment of ICA significantly reduced the amounts of IL-6, IL-10, MCP-1, IFN-\(\gamma\), TNF, and IL-12p70 in the supernatant of these cell cultures, compared with untreated cells (Fig. 7).

3.7. Effect of icaritin on the phosphorylation of MAPK in LPS-induced peritoneal macrophages

It’s well known that MAPKs are involved in the regulation of proinflammatory mediator expression in LPS-induced macrophages. Phosphorylation level of the p38, JNK and ERK MAPKs was detected by Western blotting assay (Fig. 8). Pretreatment with various concentrations of ICA significantly inhibited the LPS-stimulated increase of p38 and JNK activation in a dose-dependent manner, but not ERK, showing that regulation of p38 and JNK MAPK signaling pathway is one of the possible mechanisms for the inhibitory effect of ICA on LPS-activated macrophages.

3.8. Effect of icaritin on mortality in zymosan-induced peritonitis mice

Administration of zymosan caused severe illness in the mice, which was characterized by systemic toxicity and mortality (Fig. 9). At the end of observation period (13 days), 80% of zymosan-induced mice were dead. Treatment with ICA reduced the development of mortality caused by zymosan, only 30% of ICA was dead. ICA treatment did not cause significant changes in these parameters in sham mice (SHAM group).

3.9. Effect of icaritin on leukocytes numbers in zymosan-induced peritonitis mice

To investigate the effect of ICA on the number of immune cells in the peritoneal exudates of mice following zymosan-induced peritonitis,
absolute counting tubes for flow cytometry were employed. A marked rise of total leukocyte in peritoneal exudates was caused by the treatment with zymosan for 6 h and 24 h (Fig. 10A). The pretreatment of ICA significantly lowered total leukocyte, compared with the ZYM alone.

3.10. Effect of icaritin on NO release in zymosan-induced peritonitis mice

The release of NO in the peritoneal cavity in zymosan-induced female BALB/c mice was also examined using a Griess reagent system kit. A significant increase of NO levels in peritoneal cavity was observed. Our data showed that pretreatment of ICA significantly decreased the NO production of the peritoneal cavity at 6 h, and very significantly at 24 h, compared with the ZYM alone (Fig. 10B).

3.11. Effect of icaritin on the inflammatory cytokines production in zymosan-induced peritonitis mice

To further investigate the effect of ICA on the release of inflammatory cytokines in the peritoneal cavity collected after female BALB/c mice is induced by zymosan for 6 h, we analyzed cytokines secretion using CBA kit assay. Our data showed that pretreatment of ICA significantly reduced the amounts of IL-6, IL-10, MCP-1, and TNF in the peritoneal cavity, compared with the ZYM alone (Fig. 11).

4. Discussion

In the present investigation, we demonstrated for the first time the anti-inflammatory effect of icaritin in vitro which directly inhibited NO release, Ca²⁺ influx, phosphor-p38, phospho-JNK, and inflammatory cytokines production stimulated by LPS. The protective effect on the zymosan-induced peritonitis also provided an evidence of its anti-inflammatory activity in vivo.

It’s known that macrophages constitutively express a range of cell markers such as CD11b. CD69, one of signal transducing receptors, serves as an early activation antigen for murine macrophages [20]. To our surprise, CD69 expression of CD11b+ cells was significantly inhibited in the presence of ICA, suggesting that ICA reduced the early activation of macrophages. The elevation in intracellular calcium concentration is one of the earliest events during macrophages activation. The calcium movement is attributed to the depletion from intracellular stores induces the influx of extracellular calcium across the plasma membrane, a mechanism known as the store-operated calcium entry (SOCE) [21–23]. In macrophages, SOCE through calcium channels is the main mechanism to increase intracellular Ca²⁺ concentrations. In this paper, we found that ICA reduced calcium overload induced by LPS in the murine peritoneal macrophages, which might regard calcium influx as a potential anti-inflammatory target. Mitogen-activated
protein kinases (MAPKs) are a family of serine/threonine protein kinases that convert extracellular signals, such as stresses and growth factors, to the activation of intracellular pathways. These kinases, including the extracellular signal regulated kinase (ERK), the c-Jun amino-terminal kinase (JNK), and the p38 MAP kinase, are modulated by a phosphorylation cascade with a prototype of three proteins. Western blot analysis revealed that ICA can significantly suppress the phosphorylation of p38 and JNK to a similar extent, but not ERK.

Overproduction of NO caused by activated macrophages has been linked to the pathogenesis of a number of diverse effects, including direct cellular cytotoxicity [24] and various inflammatory processes [25]. As demonstrated in this study, we clearly found that icaritin inhibited high level of NO generated by activated macrophages in a dose-dependent manner. However, there was no obvious change on resting macrophages incubated with icaritin. Upon stimulation by LPS, macrophages can produce and release a large variety of cytokines, including inflammatory cytokines such as IL-6, IL-10, IL-12, TNF-α, IFN-γ, which modulate most of the macrophage functions and cell surface marker expression, and chemokines like MCP-1, which contribute to the recruitment of circulating monocytes within tissues [26]. Our results suggest that ICA prevents the overproduction of inflammatory cytokines and chemokine in the LPS-stimulated peritoneal macrophages, which is consistent with previous studies [18,27]. On the basis of above results, we propose the signaling pathway for ICA in LPS-stimulated murine macrophages. First, LPS acts with TLR4 and mediates PTK activation in macrophages. Second, PTK may induce PLCγ phosphorylation, which leads to production of DAG and IP3. With the increase of IP3 in the cytoplasm, intracellular pools release Ca2+, and this release may be followed by Ca2+ influx from extracellular spaces. The transient increase in intracellular free Ca2+ concentration activates PKC. Next, PKC regulates the phosphorylation of...
MAPK. Activated MAPK participates in IKK activation, and activated IKK in turn activates NF-κB by inducing the translocation of the p65/p50 dimer into the nucleus. Finally, the expression of iNOS and the production of inflammatory cytokines are elevated. ICA exerts anti-inflammatory effects in the LPS-induced macrophages by inhibiting the expression of CD69, reducing calcium overload, decreasing phospho-p38 and JNK protein expression, lowering the production of NO and inflammatory cytokines. To demonstrate the anti-inflammation activity of ICA, we also evaluated its efficacy in zymosan-induced mice peritonitis. Zymosan, a polysaccharide component derived from the cell wall of the yeast *Saccharomyces cerevisiae*, is commonly used to induce inflammation mimicking yeast infection [28,29]. After intraperitoneal injection of zymosan in mice, the animals develop acute peritonitis. The mortality in this illness was usually 80% at the end of our observation. Surprisingly, the mortality rate was markedly decreased by treatment with ICA after the zymosan administration. The results showed that ICA attenuated the inflammatory response in zymosan-induced acute peritonitis. When injected into animals, zymosan leads to infiltration of peritoneum with massive leukocytes. During its course, neutrophils (>24 h) are major cellular players [30,31]. When injected before zymosan, it significantly inhibited the accumulation of total leukocytes in mice at 6 h and 24 h. We infer that including macrophages, ICA might affect the immune function of neutrophils during the zymosan-induced peritonitis. During zymosan peritonitis, the phagocytes synthesize and release numerous mediators, including pro-inflammatory cytokines such as TNF-α, IL-6, anti-inflammatory cytokines like IL-10, chemokines e.g. MCP-1 [30–32], and nitric oxide (NO) [33]. On one hand, phagocytes eliminate pathogen particles by means of these soluble mediators; on the other hand, overproduction of inflammatory mediators at the inflamed tissue could harm the host. Our results show that pretreatment of ICA decreased the overproduction of these inflammatory mediators in zymosan-induced acute peritonitis, which was consistent with the results of our in vitro experiments.

In summary, the present study demonstrated that ICA possesses anti-inflammatory effects both in vitro and in vivo, which might be mediated through the regulation of the inflammatory cytokines and the phosphorylation of p38 and JNK. These findings suggest a novel mechanism via the MAPK pathway for the anti-inflammatory activities of ICA, providing further evidence for the pharmacological specificity of ICA.

**Conflicts of interest**

The authors declare no conflict of interests.

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**References**


