Original Research

Suppressive effects of irisflorentin on LPS-induced inflammatory responses in RAW 264.7 macrophages

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Abstract

Irisflorentin, a naturally occurring isoflavone, is an abundant active constituent in Rhizoma Belamcandae. Although some chemical studies have been reported, pharmacological actions of irisflorentin are not well studied. In this study, we demonstrate the anti-inflammatory activity of irisflorentin in lipopolysaccharides (LPS)-stimulated RAW 264.7 macrophages. Irisflorentin markedly reduces the transcriptional and translational levels of inducible nitric oxide synthase (iNOS) as well as the production of NO. Furthermore, it also significantly inhibits TNF-α, IL-1β and IL-6 at both the transcriptional and translational levels. These effects mainly act via ERK1/2 - and p38-mediated the activator protein-1 (AP-1) rather than the nuclear factor-κB (NF-κB) pathway. Thus, our study elucidates the anti-inflammatory mechanism of irisflorentin in LPS-activated RAW 264.7 macrophages.

Keywords: Irisflorentin, LPS, anti-inflammatory, RAW 264.7 macrophages, AP-1 pathway

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Introduction

The innate immune system is evolutionally conserved, and it is the first line of the defensive mechanism for protecting the host from invading microbial pathogens.[1] During microbial infections, pathogen-associated molecular patterns (PAMPs) are recognized by the host defense system, which mounts a protective response. Inflammation, an important component of innate immunity and the host response to pathogens,[2] must be precisely controlled at multiple levels, as excessive inflammation may cause shock and multiorgan dysfunction.[3]

Macrophages play a central role in host defense against pathogen microbes by recognizing bacterial components, resulting in the activation of an arsenal of anti-microbiol effectors and initiation of the inflammatory cascade.[4,5] The lipopolysaccharides (LPS) present in the outer membrane of gram-negative bacteria is regarded as a PAMP.[6] When macrophages are stimulated by LPS, there is over-production of pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), TNF-α, IL-1β, IL-6, etc., which in turn cause inflammation and damage to neighboring tissues and the macrophages themselves.[7] Thus, inhibition the production of these inflammatory mediators might be a useful therapeutic strategy in treating inflammatory diseases.[8,9]

In addition, the activator protein-1 (AP-1), an early transcriptional factor either alone or by coupling with nuclear factor-κB (NF-κB), plays an important role in regulating inflammatory response.[10,11] Many stimuli induce the binding of AP-1 to the promoter region of various genes that govern cellular processes such as inflammation, proliferation and apoptosis. Thus, AP-1 proteins are recognized as regulators of cytokine expression and are important modulators in inflammatory diseases.[12]

Rhizoma Belamcandae (RB), derived from the rhizomes of *Belamcanda chinensis* (L.) DC., is officially listed as a traditional Chinese medicine (TCM) for the treatment of inflammatory diseases such as throat disorders in Chinese Pharmacopoeia.[13] Pharmacological investigations have demonstrated that isoflavones could be major bioactive constituents of RB.[14,15] Among them, irisflorentin is one of the most abundant isoflavones in RB.[16] Previous studies mainly investigated irisflorentin's isolation and purification,[17,18] and quality and quantity evaluation,[16,19–21] but few studies focused on its pharmacological research. In this study, we explore the anti-inflammatory activity of irisflorentin in LPS-activated RAW 264.7 macrophages. Furthermore, the underlying molecular mechanisms of this effect were also investigated.

Materials and methods

Materials and reagents

The murine macrophage RAW264.7 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Irisflorentin was purchased from National Institutes for Food and Drug Control (Beijing, China) and

dissolved in DMSO at the concentration of 10 mg/mL. Mouse TNF-α and IL-6 ELISA kits were obtained from Biolegend Co. (San Diego, CA, USA). Mouse IL-1β ELISA kit was obtained from Excell Technology Co. (Shanghai, China). Antibody for iNOS and β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). Plasmids for NFκB-TA-luc, AP1-TA-luc and their controls GL6-TA were from Beyotime Institute of Biotechnology (haimen, Jiangsu, China). Antibodies for MAPKs were from Cell Signaling Technology (Danvers, CO, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were produced by Gibco BRL (Grand Island, NY, USA). The luciferase assay system and lipofectamine™ 2000 reagent were purchased from Promega Co. (Madison, Tennessee, USA) and Invitrogen (New York, CA, USA), respectively. Horseradish peroxidase-conjugated anti-rabbit or mouse IgG secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (Lancaster, Philadelphia, USA). All other reagents were of analytical grade.

Cell culture and treatment

RAW 264.7 macrophages were grown in DMEM supplemented with 10% heat-inactivated FBS and 1.0% penicillin-streptomycin solution in a humidified incubator with 5.0% CO_2 at 37°C. When performing irisflorentin treatment, irisflorentin was first added to the culture medium and then mixed thoroughly (the final concentrations of DMSO were <0.15%). The cell culture medium was replaced with the medium already mixed with irisflorentin.

Cell viability assay

The cell viability was measured by an MTT assay as previously described.[22]

NO production measurement

The level of NO production in the culture medium was assayed as previously described.[23]

Measurement of iNOS activity

The enzymatic activity of iNOS was assayed as previously described[12] with slight modifications. The cells were plated in a 25 cm^2 culture flask and incubated with LPS (10 ng/mL) for 12 h. After being washed twice by PBS, the cells were harvested and plated in a 48-well plate, and incubated in the presence or absence of irisflorentin at different concentrations for a further 12 h. The iNOS activity was assayed by measuring the nitrite level in the supernatant by Griess method. L-NAME was used as a positive control.

ELISA for TNF-α, IL-1β and IL-6

For the measurements of TNF-α, IL-6 and IL-1β, RAW 264.7 macrophages were pretreated with irisflorentin for 2 h and then stimulated with LPS (10 ng/mL) for 24 h. TNF-α, IL-6 and IL-1β in the cell supernatants were assayed using ELISA kits according to the manufacturer's instructions. The concentrations were calculated from the standard curves.

Table 1 Primers used for RT-PCR analysis

Target gene	Primer sequence
iNOS	F: 5′-CTC AGC CCA ACA ATA CAA G-3′ R: 5′-CTA CAG TTC CGA GCG TCA-3′
TNF-α	F: 5′-GCC TAT GTC TCA GCC TCT T-3′ R: 5′-GGT TGA CTT TCT CCT GGT AT-3′
IL-1β	F: 5′-ATT GTG GCT GTG GAG AAG-3′ R: 5′-TTG TGA GGT GCT GAT GTA-3′
IL-6	F: 5′-CGA TAG TCA ATT CCA GAA ACC GC-3′ R: 5′-TTG GGA GTG GTA TCC TCT GTG AAG-3′
β-actin	F: 5′-TGT TAC CAA CTG GGA CGA CA-3′ R: 5′-AAG GAA GGC TGG AAA AGA GC-3′

F: forward; R: reverse.

RNA extraction and quantitative real-time PCR

The RNA extraction and quantitative real-time PCR (*q*PCR) assays were performed as previously described.[23] The levels of iNOS, TNF-α, IL-1β and IL-6 mRNA were normalized to β-actin, a stable housekeeping gene. The primers that were used in these experiments are shown in Table 1.

Transfections and luciferase assay

RAW 264.7 macrophages were transfected with pNFκB-TA-luc, pAP1-TA-luc and their controls pGL6-TA by using the lipofectamine™ 2000 according to the manufacturer's instructions. After 24 h of transfection, the cells were pre-incubated with different concentrations of irisflorentin for 30 min and then exposed to LPS for 6 h. The cells were lysed and luciferase activity was measured using the Luciferase Assay System.

Western blot analysis

The western blot assay was performed as previously described.[23] Proteins in nucleus or cytoplasm were extracted and separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked at room temperature for 4 h with 5.0% nonfat dry milk, and then incubated with each primary antibody at 4°C overnight. After washing, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The blots were visualized using enhanced chemiluminescence, and data were analyzed using the Gel Doc EQ System (Bio-Rad).

Statistical analysis

The data were calculated as mean ± standard error of the mean (SEM) of at least three independent experiments and analyzed by a one-way ANOVA. A student *t*-test was used

when only two groups were compared. Differences were considered to be significant at $P < 0.05$.

Results

Inhibition of NO production

Cell viability analysis showed that irisflorentin did not affect the cell viability up to a concentration of 40 μM (Figure 1a). To investigate the effect of irisflorentin on inflammation, we first measured supernatant NO production in LPS-stimulated RAW 264.7 macrophages in a safe concentration range. As shown in Figure 1(b), irisflorentin inhibited the LPS-induced production of NO in a concentration-dependent manner without affecting the iNOS activity (Figure 1c).

Suppression of iNOS at the transcriptional and translational levels

We next investigated the inhibitory effects of irisflorentin on iNOS mRNA and protein levels. As shown in Figure 2, LPS stimulation of RAW 264.7 macrophages resulted in a dramatic increase in iNOS at the transcriptional and translational levels. Treatment with irisflorentin concentration-dependently inhibited the LPS-induced increase in iNOS mRNA expression and protein levels.

Suppression of pro-inflammatory cytokines

The effect of irisflorentin on the production of pro-inflammatory cytokines was examined. As shown in Figure 3(a), irisflorentin concentration-dependently suppressed the production of TNF-α, IL-1β and IL-6 in LPS-stimulated RAW 264.7 macrophages. To confirm whether the decrease in cytokine production was correlated with cytokine transcriptional levels, we analyzed the quantity of TNF-α, IL-1β, and IL-6 mRNA using *q*PCR. Irisflorentin consistently down-regulated the LPS-induced TNF-α, IL-1β, and IL-6 mRNA expressions in a concentration-dependent manner (Figure 3b).

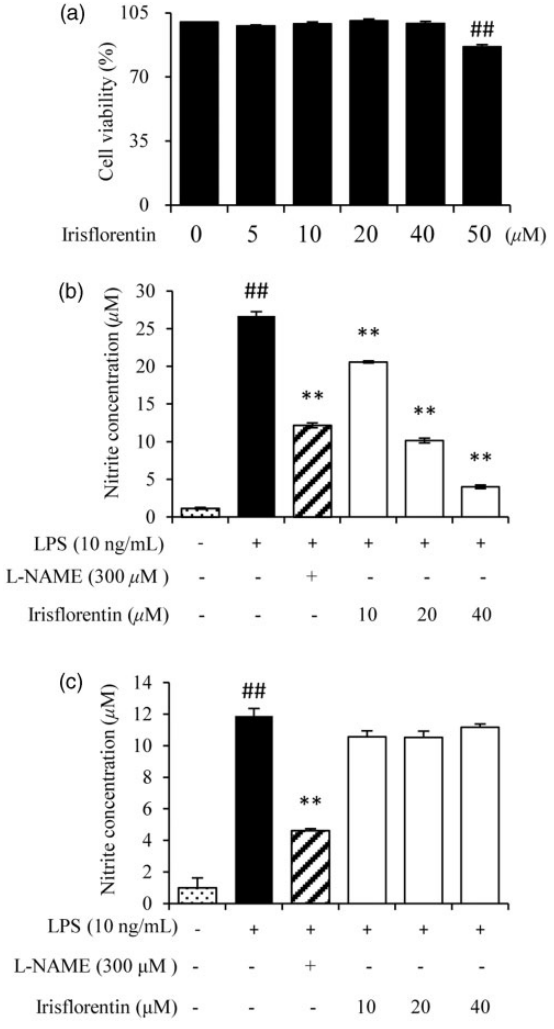

Figure 1 (a) Effect of irisflorentin on the viability of RAW 264.7 macrophages. (b) Effect of irisflorentin on LPS-induced NO production in RAW 264.7 macrophages. (c) Effect of irisflorentin on iNOS activity. $^{\#\#}P < 0.01$ vs. normal control group; $^{**}P < 0.01$ vs. LPS alone. Bars represent mean ± SEM of three independent experiments

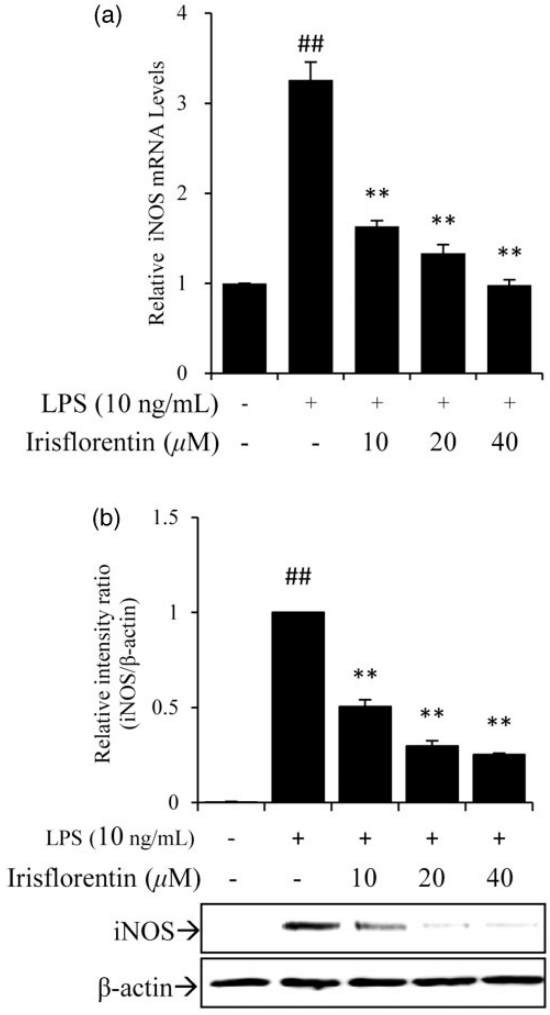

Figure 2 Effect of irisflorentin on iNOS mRNA expression (a) and protein levels (b) in LPS-stimulated RAW 264.7 macrophages. $^{\#\#}P < 0.01$ vs. normal control group; $^{**}P < 0.01$ vs. LPS alone. Bars represent mean ± SEM of three independent experiments

Effect on signal transduction pathways

Since the induction of proinflammatory mediators by LPS is known to be predominantly regulated by NF-κB and AP-1, we investigated if irisflorentin exerts anti-inflammatory action by affecting these two pathways. As shown in Figure 4, irisflorentin significantly suppressed the AP-1 signaling pathway (Figure 4a), but slightly affected NF-κB (Figure 4b). Thus, we explored the MAPKs, which primarily regulate the downstream AP-1. MAPKs are classified into at least three components: p38, ERK1/2, and JNK.

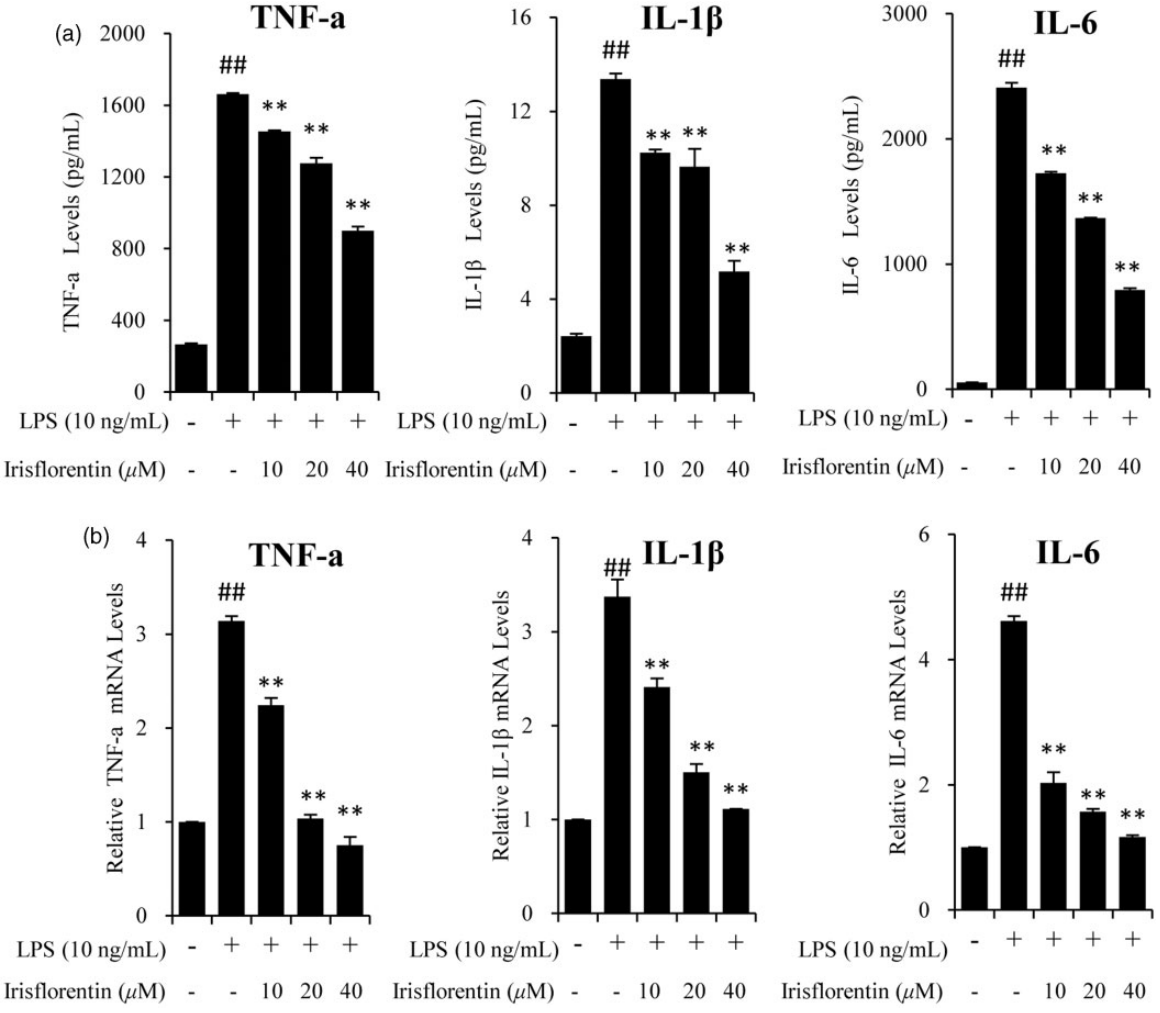

Figure 3 Effect of irisflorentin on TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW 264.7 macrophages at the translational (a) and transcriptional (b) levels. $^{\#\#}P < 0.01$ vs. normal control group; $^{**}P < 0.01$ vs. LPS alone. Bars represent mean ± SEM of three independent experiments

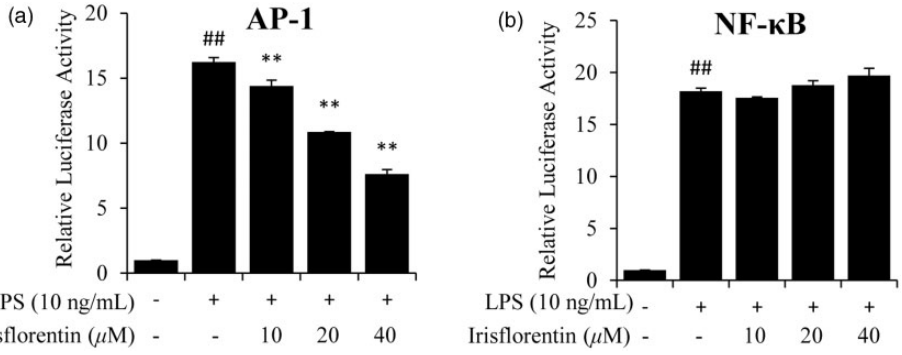

Figure 4 Effect of irisflorentin on LPS-induced AP-1 and NF-κB activities. RAW 264.7 macrophages were transfected with the luciferase reporter pAP-1-TA-luc (a) and pNF-κB-TA-luc (b). $^{\#\#}P < 0.01$ vs. normal control group; $^{**}P < 0.01$ vs. LPS alone. Bars represent mean ± SEM of three independent experiments

These kinases are ubiquitous and highly conserved mechanisms of eukaryotic cell regulation. Here, we investigated the effects of irisflorentin on phosphorylation levels of p38, ERK1/2, and JNK in LPS-stimulated RAW 264.7 macrophages. The results indicated that irisflorentin suppressed LPS-induced p38 and ERK1/2 phosphorylation in a concentration-dependent manner, but slightly affected JNK phosphorylation (Figure 5).

Discussion

RB, a TCM officially listed in Chinese Pharmacopoeia, has been widely used for the treatment of pharyngitis clinically.[20] Irisflorentin is a representative index component for the quality control of RB which has been officially listed in Chinese Pharmacopoeia.[13] Previously, many of its structurally similar isoflavones (Figure 6), such as tectorigenin,[24] genistein,[25] daidzein,[26] glycitein,[27] and biochanin A,[28] were reported to suppress proinflammatory mediators in LPS-stimulated macrophages. However, due to the minor structural differences, the anti-inflammatory mechanisms of these analogues are diverse. For example, tectorigenin[24] and genistein[25] suppress LPS-induced inflammatory responses through blocking NF-κB activation, while biochanin A[28] can inactivate both NF-κB and AP-1 signaling. In this study, we

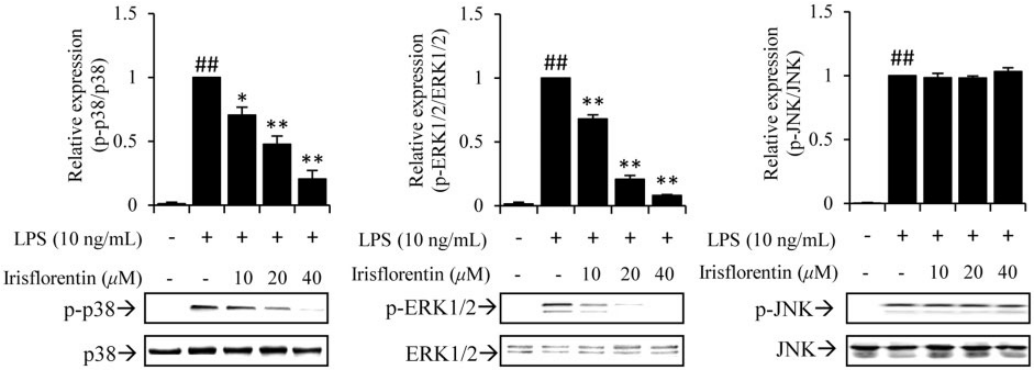

Figure 5 Effects of irisflorentin on LPS-induced ERK1/2, p38 and JNK phosphorylation levels. $^{\#\#}P < 0.01$ vs. normal control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. LPS alone. Bars represent mean ± SEM of three independent experiments

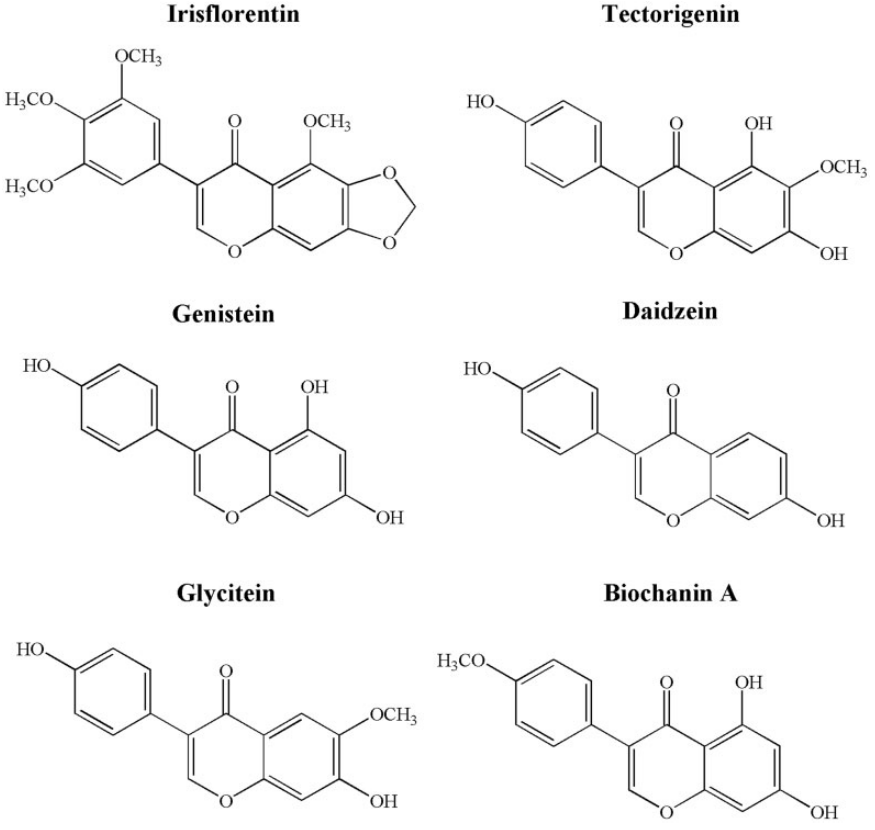

Figure 6 Chemical structures of irisflorentin and its five analogues

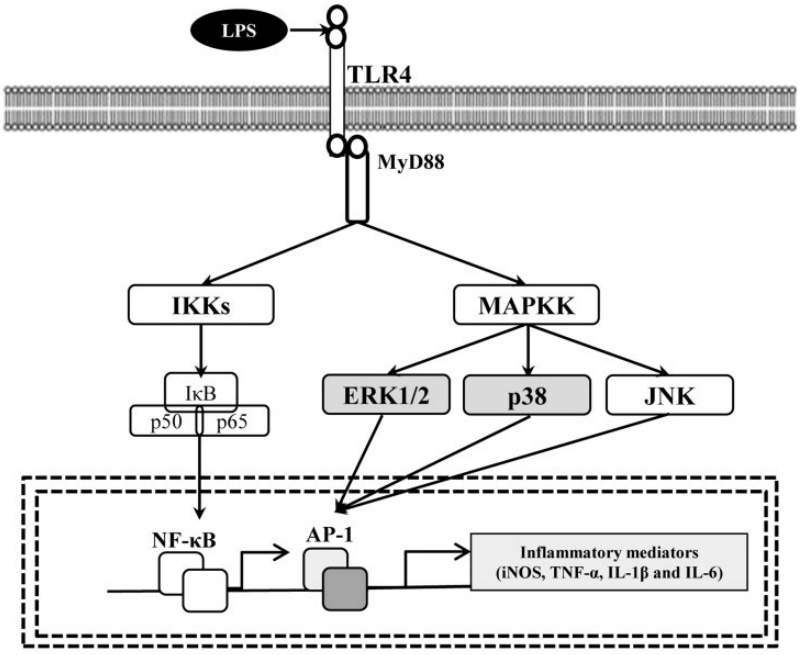

Figure 7 Proposed mechanism by which irisflorentin exerts anti-inflammatory activity in LPS-activated RAW 264.7 macrophages. The gray color indicates the targets of irisflorentin

investigated the effects of irisflorentin on LPS-induced proinflammatory mediators in RAW 264.7 macrophages. Furthermore, the underlying molecular mechanisms of these effects were also explored.

NO, a small diffusible molecular generated by iNOS in activated macrophages, is closely related to many inflammatory diseases. In this study, we found that irisflorentin could potently decrease LPS-induced NO production in RAW 264.7 macrophages without cytotoxicity. Subsequent research showed that the effect of irisflorentin on NO production was mainly attributed to the suppression of iNOS mRNA and protein levels (Figure 2), rather than the reduction of iNOS activity (Figures 1c).

Apart from NO, macrophages are also important producers of pro-inflammatory cytokines when they are stimulated by an aggression. Excessive production of these cytokines, such as TNF-α, IL-1β, and IL-6, leads to capillary leakage, vascular hemorrhage, tissue destruction, and subsequent lethal organ failure.[29] Thus, the expression of proinflammatory cytokines needs to be tightly regulated during an inflammatory response. Our results indicate that irisflorentin concentration-dependently downregulates the mRNA expression and decreases the production of TNF-α, IL-1β, and IL-6 in LPS-activated RAW 264.7 macrophages (Figure 3).

A variety of transcription factors, including NF-κB and AP-1, are known to regulate the transcription of inflammatory mediators. NF-κB transcription factors are pivotal regulators of inflammation and immunity that control the expression of important immunoregulatory genes. Independent or synergistic with NF-κB, AP-1 is a group of dimeric factors comprising members of the Jun and Fos families of DNA-binding proteins.[30] Many stimuli induce the binding of AP-1 to the promoter region of various genes that govern cellular processes such as inflammation, proliferation, and apoptosis. Thus, AP-1 proteins are recognized as regulators of cytokine expression and are important modulators in inflammatory diseases. Our results show that irisflorentin inhibits the activity of AP-1 rather than NF-κB in LPS-activated RAW 264.7 macrophages (Figure 4). Furthermore, irisflorentin inhibits the LPS-induced phosphorylation levels of p38 and ERK1/2, while JNK remains unchanged (Figure 5).

Taken together, this is the first report on the anti-inflammatory effect of irisflorentin in LPS-activated RAW 264.7 macrophages. It could inhibit the production of inflammatory mediators via suppressing the p38- and ERK1/2-mediated AP-1 pathway (Figure 7). The data in the present study support the pharmacological basis of the use of RB as a traditional herbal medicine for the treatment of pharyngitis, and thereby irisflorentin may be utilized as a beneficial anti-inflammatory candidate.

Author contributions: All authors reviewed the manuscript. YG and YQ were responsible for study design, data interpretation and manuscript writing. LF, FL, CJZ, and RLC performed the experiments. XC assisted in data analysis and critical reading of the manuscript.

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