

Inhibitory function of Shikonin on MRGPRX2-mediated pseudo-allergic reactions induced by the secretagogue

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ARTICLE INFO

Keywords:

Shikonin

Mrgprx2

Mast cells

Pseudo-allergic reactions

ABSTRACT

Background: Mast cells (MCs) are crucial effectors in allergic disorders by secreting inflammatory mediators. The Mas-related G-protein-coupled receptor X2 (Mrgprx2) was shown to have a key role in IgE-independent allergic reactions. Therefore, potential drug candidates that directly target Mrgprx2 could be used to treat pseudo-allergic diseases. Shikonin, an active ingredient derived from *Lithospermum erythrorhizon* Sieb. et Zucc has been used for its anti-inflammatory properties since ancient China.

Purpose: To investigate the inhibitory effects of Shikonin on IgE-independent allergy both in vitro and in vivo, as well as the mechanism underlying its effects.

Methods/study designs: The anti-anaphylactoid activity of Shikonin was evaluated in PCA and systemic anaphylaxis models, Calcium imaging was used to assess intracellular Ca^{2+} mobilization. The release of cytokines and chemokines was measured using enzyme immunoassay kits. Western blot analysis was conducted to investigate the molecules of PLC γ -PKC-IP3 signaling pathway. The analytical method of surface plasmon resonance was employed to study the interaction between Shikonin and potential target protein Mrgprx2.

Results: Shikonin can suppress compound 48/80 (C48/80)-induced PCA, active systemic anaphylaxis, and MCs degranulation in mice in a dose-dependent manner. In addition, Shikonin reduced C48/80-induced calcium flux and suppressed LAD2 cell degranulation via PLC γ -PKC-IP3 signaling pathway. Moreover, Shikonin was found to inhibit C48/80-induced Mrgprx2 expression in HEK cells, displaying specific interactions with the Mrgprx2 protein.

Conclusion: Shikonin could be a potential antagonist of Mrgprx2, thereby inhibiting pseudo-allergic reactions through Ca^{2+} mobilization.

Introduction

Anaphylactoid reactions occur rapidly and systemically, affecting one or more organ systems, such as the respiratory tract (laryngeal edema and bronchospasm), the cardiovascular system (hypotension, syncope, and arrhythmias), and the epidermis and dermis (urticaria and angioedema); generally, organs where mast cells (MCs) reside in relative abundance, are affected (Estelle and Simons, 2008; Sampson et al., 2006). MCs are crucial effectors of anaphylactoid reactions and, upon activation, can quickly induce the secretion of pre-stored mediators such as histamine, serotonin, proteoglycans, neutral proteases, chemokines, and cytokines (Amin, 2012; Frenzel and Hermine, 2013; Galli and Tsai, 2012; Moon et al., 2014).

Various basic cationic molecules, such as LL37, Substance P and Compound 48/80 (Lansu et al., 2017; Lu et al., 2017; McNeil et al., 2015), can induce MC degranulation via the Mas-related G-protein-coupled receptor X2 (Mrgprx2) in humans and Mrgprb2 (the ortholog of human Mrgprx2) in rodents (McNeil et al., 2015), which is involved in IgE-independent allergy or pseudo-allergy (Tatemoto et al., 2006). Subramanian et al. described the possible role of Mrgprx2 in drug-induced anaphylactoid reactions, neurogenic inflammation, pain, itchiness, and chronic inflammatory diseases (Subramanian et al., 2016a). In consequence, small-molecule inhibitors targeting Mrgprx2 might be an effective treatment option for MC-activation induced anaphylactoid diseases.

Zicao (purple gromwell), the dried root of *Lithospermum*

Abbreviations: C48/80, compound 48/80; DMSO, dimethyl sulfoxide; HDC, histidine decarboxylase; IL, interleukin; LAD2, Laboratory Allergic Disease 2; MCP, monocyte chemotactic protein; Mrgprx2, The Mas-related G-protein-coupled receptor X2; TNF, tumor necrosis factor

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<https://doi.org/10.1016/j.phymed.2019.153149>

Received 29 July 2019; Received in revised form 13 November 2019; Accepted 11 December 2019

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erythrorhizon Sieb. et Zucc is a commonly used herbal medicine in China and other countries (Chen et al., 2002). *Lithospermum radix* extract is reported to inhibit C48/80-induced both histamine release and the production of inflammatory cytokines in MCs (Kim et al., 2007). Shikonin is a major component of zicao and has various pharmacological properties, including anti-inflammatory and anti-cancer properties, as well as the ability to mediate cellular and humoral immunity. Lu et al. reported that Shikonin could inhibit inflammation *in vivo* as well as inhibit the release of the inflammatory mediator TNF- α in rat macrophage cells (Lu et al., 2011). The inhibitory effect of Shikonin on IgE-dependent MCs activation and allergic reactions in a murine model of asthma has also been reported (Lee et al., 2010; Wang et al., 2017, 2014). In addition, Acetylshikonin was found to suppress histamine release from rat peritoneal mast cells stimulated with C48/80 (Wang et al., 1995).

However, there are few studies that examine the effects of Shikonin in IgE-independent allergic reactions. As such, the aim of the present study is to investigate the anti-pseudo-allergy effects of Shikonin both *in vitro* and *in vivo*, as well as the mechanism underlying its effect.

Materials and methods

Drugs and reagents

Shikonin (purity $\geq 98\%$) (JOT-10009) was supplied by Chengdu Pufei De Biotech Co. Ltd. (Chengdu, Sichuan, China). The drug was dissolved in DMSO as a stock, and then diluted with medium to the experimental concentrations to keep DMSO concentration below 0.05% *in vitro* experiments. Shikonin was dissolved in vehicle solution (0.5% DMSO with saline) to obtain an appropriate concentration for intraperitoneal injection *in vivo* experiments. Compound 48/80 was purchased from Sigma-Aldrich (St. Louis, USA). Fluo-3, AM ester, and Pluronic F-127 were from Biotium (California, USA). Tyrode's solution was prepared fresh on the day of use (6.954 g/l NaCl, 0.353 g/l KCl, 0.282 g/l CaCl₂, 0.143 g/l MgSO₄, 0.162 g/l KH₂PO₄, 2.383 g/l HEPES, 0.991 g/l glucose, and 1 g/l BSA, pH 7). The p-nitrophenyl N-acetyl- β -D-glucosamide and Triton X-100 were purchased from Sigma Aldrich (Shanghai, China). Stop buffer contained a mixture of 0.1 M sodium carbonate and sodium bicarbonate (pH 11). All aqueous solutions were prepared using ultrapure water produced by MK-459 Millipore Milli-Q Plus ultra-pure water system.

Animals

Adult male C57BL/6 mice (18–22 g) were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Animals were housed in individual cages in a large colony room, with free access to water, and fed a standard dry chow twice a day. The breeding environment was 20–25 °C, with a relative humidity of 40% on a 12 h light/dark cycle. The mice were randomly divided into the following groups (6 mice per group): vehicle, control, and the experimental group. All experiments involving equal treatments of animals were conducted by experimenter blind to the experimental conditions.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocols for using mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011-0045). All animals were operated under chloral hydrate anesthesia.

Hind paw swelling and extravasation

Adult male mice weighing 18–22 g, were anaesthetized with an

intraperitoneal injection of 0.2 ml 1% Phenobarbital. Fifteen minutes after the induction of anesthesia, each mouse was intraperitoneally administered (i.p.) Shikonin (0, 2.5, 5, 10 mg/kg) and intravenously injected (i.v.) with 0.2 ml of 0.4% Evans blue in saline. A Vernier caliper was used to measure the thickness of the paw before any injection and as needed during the experiments (Schafer et al., 2013). 30 min later, 30 μ g/ml C48/80 in saline was injected into the left paw and saline only into the right paw as a blank control. After 15 min, paw thickness was measured again and recorded. Mice were then killed by decapitation, and a photo of each paw was taken. Paw tissues were collected, dried for 24 h at 50 °C, and weighed. Evans blue was extracted by a 24 h incubation in 500 μ l acetone-saline (7:3) at 37 °C. Tissues were cut into pieces, placed for 10 min in an ultrasonic machine, centrifuged for 20 min at 3000 rpm. The supernatant was equally distributed in 200 μ l aliquots into 96 well cell culture plates, and the OD was read at 620 nm using a spectrophotometer. For studies using drug substances, mice were intraperitoneally administered 0.2 ml vehicle solution (0.5% DMSO with saline) and injected with 5 μ l of a 30 μ g/ml solution of C48/80 as a negative control.

Histological analysis

Anaesthetized mice were i.p. injected with Shikonin (0, 2.5, 5, 10 mg/kg). 30 mins after i.p., each mouse was injected with 5 μ l of 30 μ g/ml of C48/80 in the left paw; saline and C48/80 were used as vehicle and negative controls, respectively. Fifteen minutes later, the injection site of the skin in paw was excised, washed with PBS and fixed with 4% formaldehyde for 48 h and subjected to H&E staining. After staining the slides were dried at 37 °C for 30 min and pre-incubated in blocking solution (10% normal goat serum (v/v), 0.2% Triton X-100 (v/v) in PBS, pH 7.4) for 2 h at 25 °C, followed by incubation with 1/500 FITC-avidin for 45 min. Sections were washed 3 times with PBS, and a drop of Fluoro-mount G (Southern Biotech, AL, U.S.A) was added. Images were taken immediately using a confocal scanning laser microscope (Nikon, Tokyo, Japan).

Body temperature

All the mice were housed in the procedure area the day before the injection. The mice were randomly divided into 2 groups ($n = 6$ each group). The mice were then placed in a restrainer. Mice were i.p. injected with Shikonin (0, 10 mg/kg), followed by injection into the tail veins with C48/80 (2.5 mg/kg). The body temperature was recorded using a biological function experimental system, in which a probe was inserted into the anus of the mice for 30 min (Wang et al., 2018b).

Analysis of mouse serum

Mice were i.p. injected with Shikonin (0, 2.5, 5, 10 mg/kg), followed by injection into the tail veins with C48/80 (2.5 mg/kg). After 30 min, blood was collected from retroorbital plexus and centrifuged to get serum for histamine sample (Kaida et al., 2010). After 6 h, serum was collected by the same method for cytokine samples (Che et al., 2019). Since, MCs activation is accompanied by the fast external release of granule-associated stored mediators (histamine) and by the production of an array of cytokines at later times (McNeil et al., 2015). Mouse TNF- α , MCP-1, and IL-8 ELISA Kits were purchased from w BIOTECH CO., LTD. (Beijing, China) and used to measure the levels of TNF- α , MCP-1 and IL-8 levels according to provided instructions.

Cell lines

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). The MRGPRX2 receptor was confirmed to be present on LAD2 cells (McNeil et al., 2015). Cells were maintained in StemPro™ – 34 medium supplemented with 1% StemPro™ nutrient supplement, 1:100 penicillin-streptomycin, 2 mM l-glutamine, and 100 ng/ml human stem cell

factor in 5% CO₂ at 37 °C. The culture medium was replaced per week and cells maintained at a density of 2×10^6 cells/ml. Mrgprx2-HEK cells were kindly provided by Professor Xinzhong Dong from Johns Hopkins University (MD, USA) and were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin–streptomycin (HyClone, UT).

Cytotoxicity assays

Cell viability was determined using Abbkine-Cell Counting Kit assays (California, USA). LAD2 cells were seeded into 96-well plates at a density of 5×10^3 cells per well, and then treated with 10 µl of Shikonin at different concentrations (0, 12.5, 25, 50, 100, 200 µM) for 12 h. Next, 10 µl of Cell Counting Kit solution was added to each well followed by incubation for 2 h. Further, the relative cell viability was assessed by detection of absorbance at 450 nm using a microplate reader (Bio-Rad, Carlsbad, CA, USA).

β-hexosaminidase release assay

LAD2 cells (1×10^6 cells per well) were incubated with tyrode's solution containing Shikonin (1, 2, 4 µM) for 30 min at 37 °C in 5% CO₂. Blank and negative controls were treated with tyrode's solution alone under the same incubation conditions. After the buffer was removed, tyrode's solution containing 30 µg/ml C48/80 was added to the Shikonin-treated cells and negative control, and tyrode's solution only was added to blank control. In order to analyze the total β-hexosaminidase content, cells were lysed with 0.1% Triton X-100 in tyrode's solution. The β-hexosaminidase released into the supernatants and in cell lysates was quantified by hydrolysis of p-nitrophenyl N-acetyl-β-D-glucosamide in 0.1 M citric acid/sodium citrate buffer (pH 4.5) for 90 min at 37 °C. The reaction was developed by adding stop buffer (0.1 M sodium carbonate/ sodium bicarbonate, pH 11). The absorbance was measured at 405 nm using a microplate spectrophotometer and the percentage of β-hexosaminidase release was calculated as follows: absorbance of culture supernatant at 405 nm $\times$ 100/absorbance of total cell lysate supernatant at 405 nm.

Histamine release assay

Histamine release assay was carried out using LC-ESI-MS/MS method and Histamine (HA) and formic acid (mass spectrometry grade) were purchased from Sigma, Histamine·2HCl (A, A, B, B-D4, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA), HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, USA). Mass spectrometry grade formic acid was from Sigma. In the applied LC-ESI-MS/MS method, an LCMS 8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used. Histamine was evaluated on the system with an HILIC column (Venusil HILIC, 2.1 mm $\times$ 150 mm, 3 µm, Agela Technologies, Tianjin, China), and an isocratic elution with acetonitrile–water containing 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 ml/min.

HDC (histidine decarboxylase) assay

LAD2 cells (1×10^6 cells per well) were treated with Shikonin and C48/80 for 30 min. Cells were lysed in potassium phosphate buffer (HDC reaction buffer, 0.1 M, pH 6.8) containing dithiothreitol (0.2 mM), pyridoxal (0.01 mM)-phosphate (5%), polyethylene glycol (Mr = 300, 1%), and phenylmethylsulfonyl fluoride (100 µg/ml) according to a previous report (Han et al., 2016). After a centrifugation, the supernatant was collected. HDC activity ELISA kit was purchased from w BIOTECH CO., LTD. (Beijing, China) and used to measure the activity of HDC in supernatant.

Chemokine release assay

LAD2 cells (1×10^6 cells per well) were incubated with tyrode's

solution containing Shikonin (1, 2, 4 µM) and 30 µg/ml C48/80 for 6 h at 37 °C, tyrode's solution containing 30 µg/ml C48/80 was added to negative control and tyrode's solution only was added to blank control. TNF-α, MCP-1, MIP1-β and IL-8 ELISA Kits were purchased from w BIOTECH CO., LTD. (Beijing, China) and used to measure the levels of TNF-α, MCP-1, MIP1-β and IL-8 levels in supernatants according to provided instructions.

Intracellular Ca²⁺ mobilization assay

The incubation buffer consisted of 0.5 µl Fluo-3, 2 µl Pluronic F-127, and 997.5 µl calcium imaging buffer (CIB; 125 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 0.6 mM MgCl₂, 10 mM HEPES, 20 mM glucose, 1.2 mM NaHCO₃, 20 mM sucrose, pH 7.4). Different concentrations of Shikonin (1, 2, 4 µM) were prepared in incubation buffer, an incubation buffer without Shikonin was used as a negative control.

LAD2 cells or Mrgprx2-Hek293 cells (1×10^4 cells per well) were incubated with the required incubation buffer for 30 min at 37 °C, followed by imaging. For calcium imaging, one photo per second was taken under the blue light (200 $\times$ magnification). Cells were identified as responding if the [Ca²⁺] ion by at least 50% after injected the substances.

Western blot analysis

Protein was extracted from LAD2 cells treated with 30 µg/ml C48/80 and 0, 1 µM, 2 µM and 4 µM Shikonin for 1 h. Proteins were immunoblotted with the following antibodies: anti-PLCγ1, anti-phosphorylated-PLCγ1 (P-PLCγ1, Ser1248), anti-IP3R, anti-phosphorylated-IP3R (P-IP3R, Ser1756), anti-PKC, anti-phosphorylated-PKC (P-PKC, Ser1756), anti-P38, anti-phosphorylated-P38 (P-P38, Thr180/Tyr182), anti-AKT, anti-phosphorylated-AKT (P-AKT, Ser473), anti-ERK1/2, anti-phosphorylated-ERK1/2 (P-ERK1/2, P-p44/42 MAPK (ERK1/2) [Thr202/Tyr204], or anti-GAPDH antibodies. GAPDH was used as a reference for relative protein level.

Molecular docking

To investigate the interactions between the receptor and ligand, molecular docking studies were conducted using the Sulflex-Dock Mode of Sybyl-X program package (New Tripes International, St. Louis, USA). The docking model of Mrgprx2 used in the study was based on the homology model of Mrgprx2 reported by Dr. Bryan Roth (Lansu et al., 2017).

Surface plasmon resonance (SPR)

Mrgprx2 protein with 6 $\times$ his tag (30 µg/ml) was fixed on the NTA sensor chip by capture-coupling, then 0.05, 0.5, 2.5, 10 µmol/l of Shikonin were injected sequentially into the chamber coupled with PBS running buffer, the interaction of Mrgprx2 with the small molecules fixed was detected by Open SPR™ (Nicoya Lifesciences, Waterloo, Canada) at 25 °C. The binding time and disassociation time were both 250 s, the flow rate was 20 µl/s, the chip was regenerated with Hydrochloric acid (pH 2.0). A one to one diffusion corrected model was fit to the wavelength shifts corresponding to the varied drug concentration. The data was retrieved and analyzed with TraceDrawer software (Yang et al., 2018).

Statistical analysis

Data are expressed as mean $\pm$ standard error of mean (S.D.), and were statistically analyzed using analysis of variance (ANOVA). Two-tailed tests were used for comparisons between two groups, and differences were considered significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

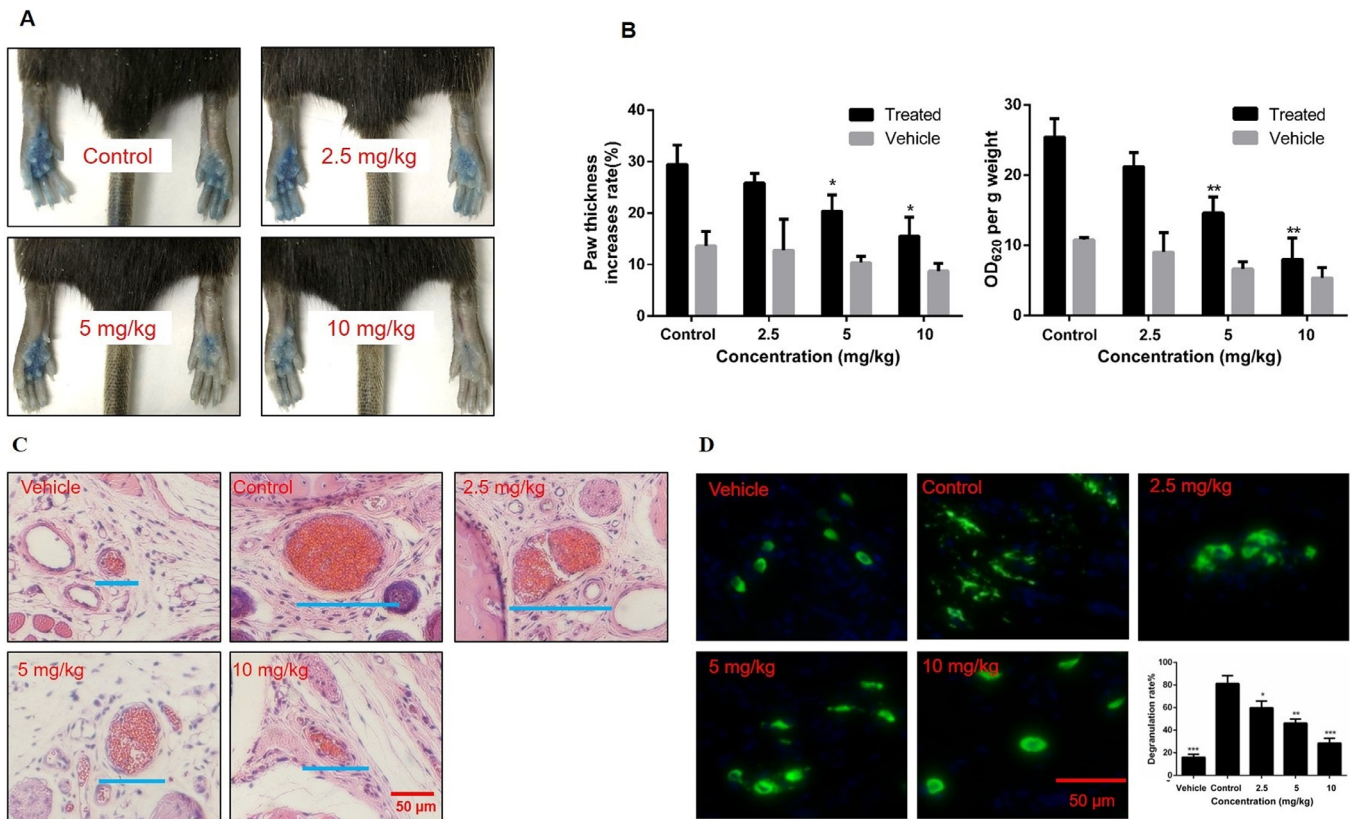


Fig. 1. Shikonin suppresses C48/80-induced local inflammation by inhibiting mast cell degranulation. Different Shikonin concentrations (0, 2.5, 5, 10 mg/kg) were intraperitoneally administered to mice. At 30 min post-treatment, 30 μ g/ml C48/80 in saline was injected into the left paw, while saline was simultaneously injected into the right paw as a vehicle control. A. The degree of swelling and Evans blue exudation in mice hind paws were analyzed 15 min after injection. B. H&E staining of the paw skin. C. Avidin staining of the paw skin. Data are presented as means $\pm$ SD ($n = 6$). D. Two-tailed unpaired Student's *t*-test was used to determine statistical significance. Statistical significance was defined as $p < 0.05$ (* $p < 0.01$, and *** $p < 0.001$ compared with the control). Scale bar = 50 μ m.

Results

Shikonin inhibited C48/80-induced local inflammation

We established a mouse model with C48/80-induced skin inflammation, which was previously shown to be IgE-independent, in order to evaluate the effect of Shikonin (Kaida et al., 2010). Intraperitoneal administration of Shikonin before C48/80 injection into mouse hind paw decreased swelling and Evans blue exudation in a dose-dependent manner, which indicated that Shikonin has a significant suppression effect on local inflammation (Fig. 1A). The decrease in local extravasation was caused by a decrease in hemangiectasis (Fig. 1B). Furthermore, C48/80-induced MC degranulation in the paw was significantly reversed following Shikonin administration (Fig. 1C). Taken together, these results indicate that Shikonin attenuates the local inflammation through the inhibition of MCs degranulation.

Shikonin inhibited C48/80-induced systemic anaphylaxis

In order to assess the contribution of Shikonin in anaphylaxis reactions, body temperature (BT) was measured (Fig. 2A). No mice died during the assay. At 21 min after C48/80 injection, BT decreased by 3.02 ± 0.25 °C. However, when pretreated with Shikonin, the effect of C48/80 on BT was significantly reduced. The mice that received Shikonin only showed a 1.5 ± 0.14 °C reduction in BT. Moreover, 30 min after C48/80 injection, the reduced BT of Shikonin-treated mice returned to approximately normal values (0.55 ± 0.21 °C lower than the BT measured at the beginning of the experiment). In contrast, the reduced BT of the control group which received vehicle solution did not

recover (2.65 ± 0.23 °C lower than the BT measured at the beginning of the experiment).

We also observed that Shikonin pre-treatment reduced the levels of plasma histamine and cytokines (TNF- α , IL-8, and MCP-1) in mice injected with C48/80 (Fig. 2B–E). As such, these results suggest that Shikonin could inhibit C48/80-induced systemic anaphylaxis.

Shikonin reduced C48/80-induced LAD2 cells activation

The concentrations of Shikonin used in this study had little effect on the viability of LAD2 cells (Fig. 4A). Therefore, LAD2 cells were treated with a non-cytotoxic concentration of Shikonin in order to investigate whether it could arrest MC activation. Calcium mobilization is an essential event that initiates MC activation and histamine production. Therefore, in order to determine the effect of Shikonin on calcium flux and the activation of MCs, LAD2 cells were pre-treated with Shikonin for 30 min and then loaded with Fluo-3 AM. Indeed, after Shikonin treatment, both the calcium flux observed and the responding LAD2 cells were decreased in a dose-dependent manner (Fig. 3).

The release of β -hexosaminidase and histamine were assayed in order to determine whether Shikonin could inhibit C48/80-induced MCs degranulation. Our results showed that β -hexosaminidase release was inhibited in a dose-dependent manner (IC₅₀ value was 1.32 ± 0.085 μ M) (Fig. 4B, C). Likewise, histamine secretion was reduced in Shikonin-treated LAD2 cells (Fig. 4D). Whereas the activity of HDC (Fig. 4E) has little variation among each group. In addition, Shikonin significantly reduced the secretion of cytokines, such as TNF- α , MCP-1, MIP-1 β , and IL-8 (Fig. 4F–I) in LAD2 cells.

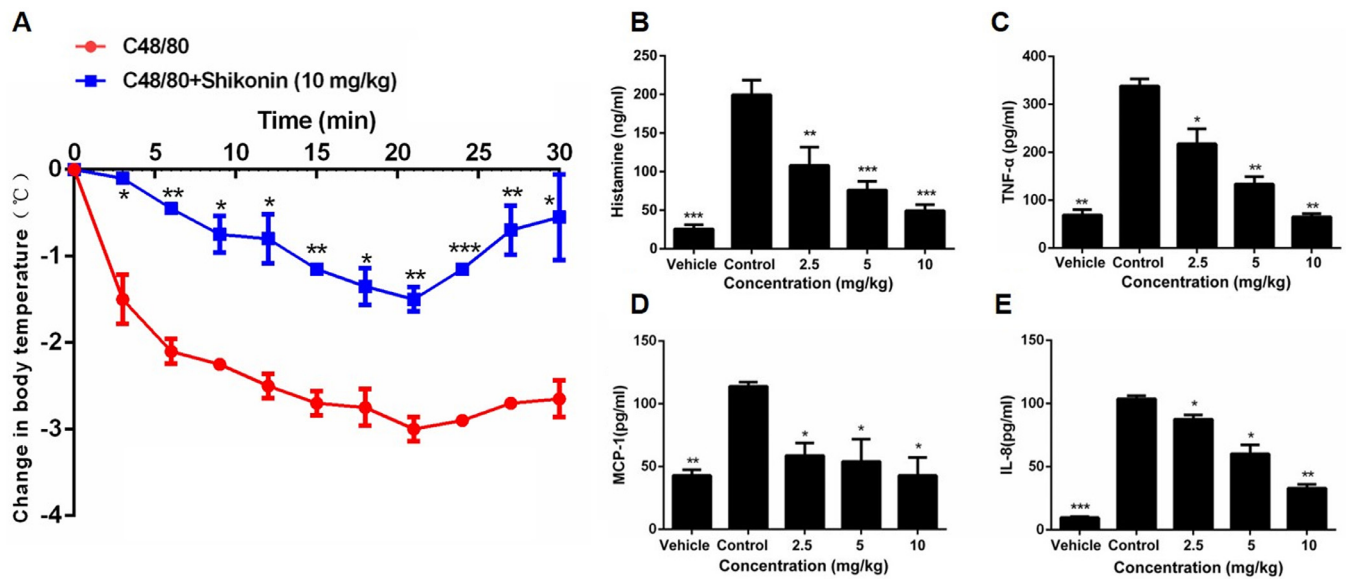


Fig. 2. Shikonin inhibits C48/80-mediated systemic anaphylactic reaction. A. Mice were pre-treated with Shikonin (0, 10 mg/kg). The change in body temperature at 30 min after injection with C48/80 was measured. B-E. Mice were pretreated with different Shikonin concentrations (0, 2.5, 5, 10 mg/kg). The serum levels of histamine (B), TNF-α (C), MCP-1 (D), and IL-8 (E) were measured after injection with C48/80 into the tail vein. Data are presented as means $\pm$ SD ($n = 6$). Two-tailed unpaired Student's t -test was used to determine statistical significance. Statistical significance was defined as $p < 0.05$ (* $p < 0.01$, and *** $p < 0.001$ compared with the control).

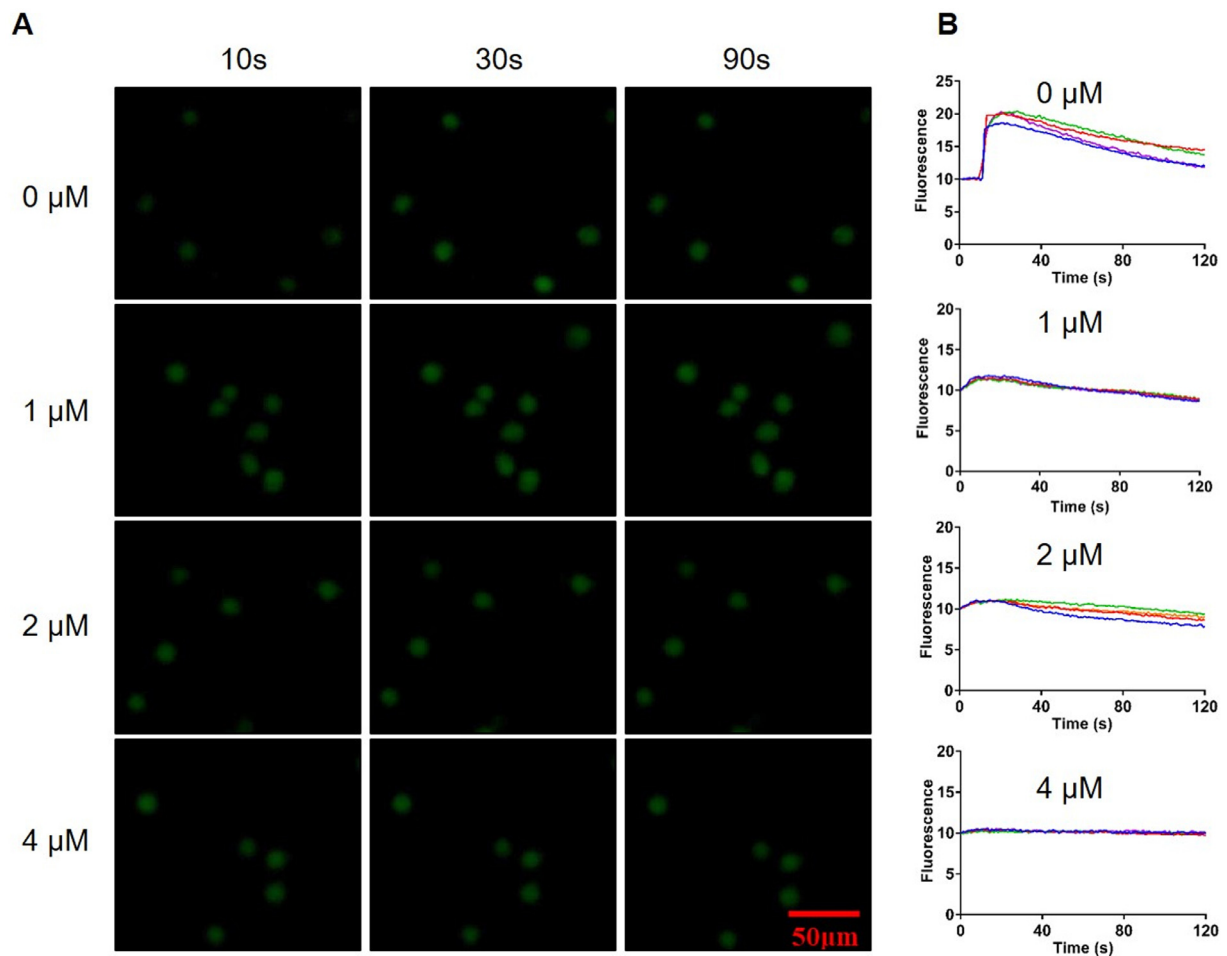


Fig. 3. Shikonin reduces C48/80-induced calcium (Ca^{2+}) influx in mast cells. LAD2 cells were incubated with different Shikonin concentrations (0, 1, 2, 4 μ M). A. Representative Fluo-3 fluorescence images of LAD2 cells showing changes in $[\text{Ca}^{2+}]_i$ after treatment with C48/80. B. Changes in the calcium influx in LAD2 cells after treatment with C48/80. Each color line represents an individual cell. Scale bar = 50 μ m.

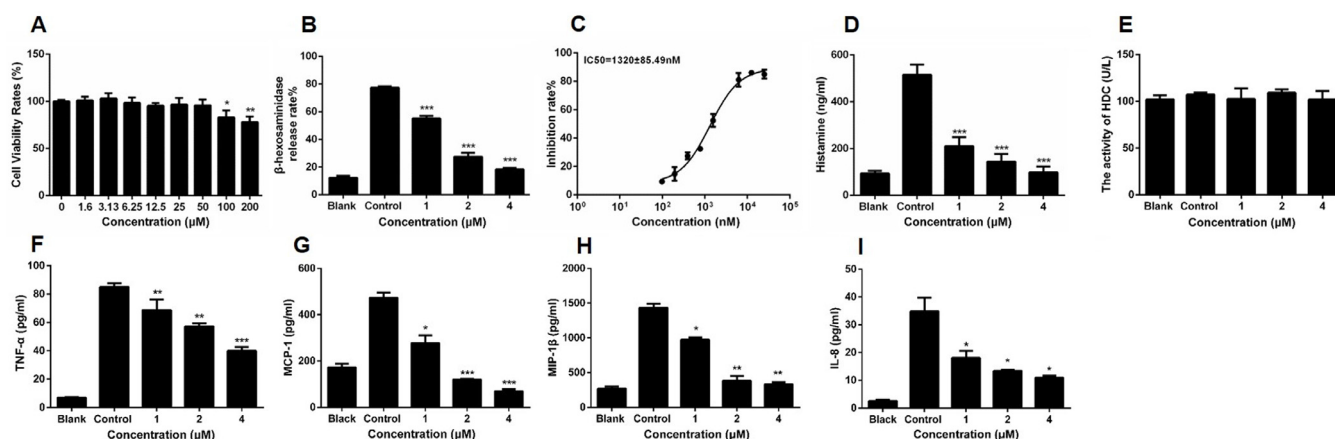


Fig. 4. Shikonin inhibits C48/80-induced degranulation, cytokines and chemokines secretion in LAD2 cells. C48/80 (30 μg/ml) was used as an agonist for LAD2 cells and as the negative control. Tyrode's solution was used as a blank control. A. The viability of LAD2 cells treated with Shikonin was investigated after incubating for 24 h. B. Release of β-hexosaminidase from LAD2 cells treated with Shikonin (0, 1, 2, 4 μM) and C48/80 (30 μg/ml) for 30 min. C. The IC₅₀ value of Shikonin for β-hexosaminidase secretion. D. Release of histamine from LAD2 cells treated with Shikonin and C48/80 for 30 min. E. The activity of HDC in LAD2 cells treated with Shikonin and C48/80 for 30 min. F-I. Release of TNF-α (F), MCP-1 (G), MIP-1β (H) and IL-8 (I) from LAD2 cells treated with Shikonin and C48/80 for 6 h. Data are presented as means ± SD (*n* = 3). Two-tailed unpaired Student's *t*-test was used to determine statistical significance. Statistical significance was defined as *p* < 0.05 (**p* < 0.05, ***p* < 0.01, and ****p* < 0.001 compared with the control).

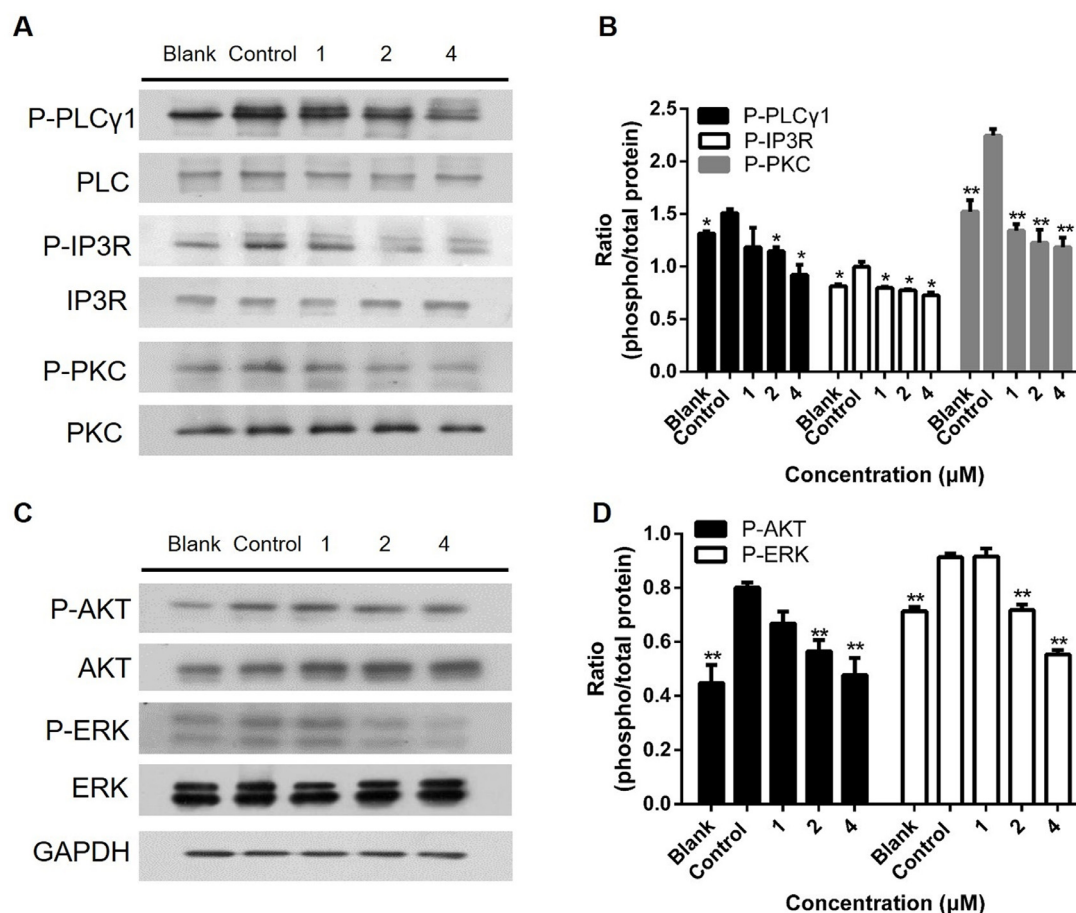


Fig. 5. Shikonin attenuates C48/80-induced phosphorylation of PKC, PLCγ1, IP3R, AKT, and ERK1/2 in LAD2 cells. A and C. Western blot analysis of the expression levels of P-PKC, PKC, P-PLCγ1, PLCγ1, P-IP3R and IP3R (A) P-AKT, AKT, P-ERK1/2, ERK1/2 (C), in LAD2 cells treated with Shikonin (0, 1, 2, 4 μM) and 30 μg/ml C48/80 for 1 h. B and D. Quantification of western blot by densitometric analysis. Data are presented as means ± SD (*n* = 3). Two-tailed unpaired Student's *t*-test was used to determine statistical significance. Statistical significance was defined as *p* < 0.05 (**p* < 0.05 and ***p* < 0.01 compared with the control).

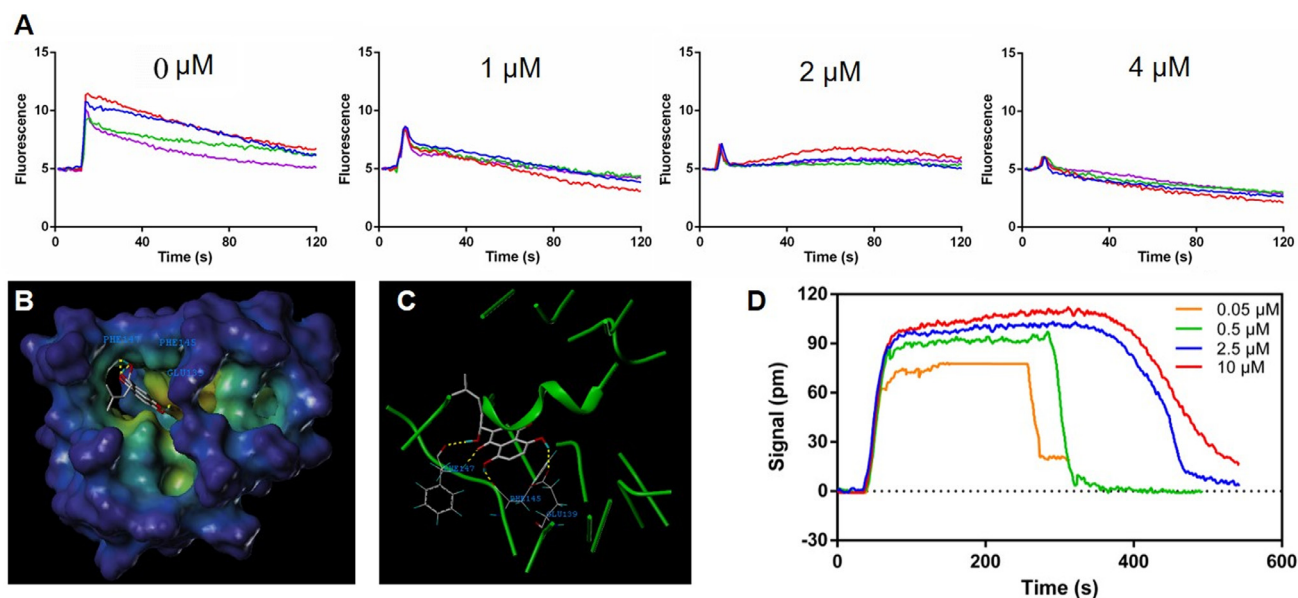


Fig. 6. Co-interaction between Shikonin and Mrgprx2. A. Shikonin reduced C48/80-induced calcium influx in Mrgprx2-HEK293 cells. (Example traces showing changes in $[Ca^{2+}]_i$, as measured by Fluo-3 imaging, each color line represents an individual cell.) B-C. Schematic diagram of the binding between Shikonin and the Mrgprx2 protein determined via a molecular docking assay. Sphere space field model of Shikonin and Mrgprx2 (B). Ribbon model of Shikonin and Mrgprx2 (C). D. The binding curves of Shikonin on the Mrgprx2-NTA sensor chip by SPR.

Shikonin decreases calcium fluctuations in mast cells via the PLC γ -PKC-IP3 signaling pathway

The phosphorylation of PKC, PLC γ 1 and IP3R (Fig. 5A, B) were significantly increased in the group treated with C48/80 when compared to the blank group. However, this effect was reduced by Shikonin treatment in a dose-dependent manner. In addition, the levels of phosphorylated AKT and ERK1/2 (Fig. 5C, D) were decreased by Shikonin treatment, when compared with those of LAD2 cells treated with C48/80 alone.

Shikonin serves as an Mrgprx2 specific inhibitor

We established a HEK cell line (Mrgprx2-HEK293) with stable Mrgprx2 expression and investigated the effect of Shikonin on C48/80-induced Ca^{2+} flux increase. Mrgprx2-HEK cells pre-treated with Shikonin showed a dose-dependent inhibition of cell activation (Fig. 6A).

Molecular docking studies were used to investigate ligand-protein interactions between Mrgprx2 and Shikonin. The sphere space field model (Fig. 6B) showed that Shikonin has a good fit within the active cavity of Mrgprx2. Furthermore, Shikonin interacted with Mrgprx2 by forming four hydrogen bonds (Fig. 6C). Three hydroxyl groups and a carbonyl group of Shikonin formed four hydrogen bonds with the amino acids PHE147, PHE145, and GLU139 within the active center of Mrgprx2, with bond lengths of 2.12 Å, 1.95 Å, 2.12 Å, and 2.04 Å, respectively.

In order to further confirm that Shikonin can bind to Mrgprx2, SPR analysis was performed. The results showed that Shikonin could bind the Mrgprx2 protein, with the K_D value for Shikonin calculated by TraceDrawer™ being 1.78e-6 mol/l (Fig. 6D).

Discussion

In the current study, we report that Shikonin inhibited MCs activation *in vivo* and *in vitro* by regulating an Mrgprx2-mediated pathway, which contributes to its inhibition effects on pseudo-allergic reactions.

MCs are effector cells that mediate immediate hypersensitivity and allergic diseases (Galli et al., 2008). Mrgprx2 is a G-protein coupled receptor (GPCR) expressed on MCs that has been implicated in pseudo-allergic reactions in humans, and it allows MCs to degranulate in

response to several drugs, including morphine, Icatibant or Cetrorelix. (Espinosa and Valitutti, 2018). Recently, Wang et al. reported that Saikosaponin A, a potential inhibitor of Mrgprx2, could lead to a therapeutic suppression of pseudo-allergic reactions (Wang et al., 2018a). However, Mrgprx2 inhibitors have not been previously used in order to treat allergic diseases. As such, the search for novel anti-allergy agents derived from medicinal plants is highly necessary.

Skin MCs are unique immune cells that constitutively express Mrgprx2. Histamine (Pastwinska et al., 2017; Xie and He, 2005), tryptases, and chymases can be swiftly released upon degranulation, allowing MCs to quickly affect tissue homeostasis (vascular permeability, vasomotricity, matrix remodeling, etc.) and to participate in inflammatory processes (Espinosa and Valitutti, 2018). In the present study, we used a mouse model of pseudo-allergic reactions to explore the anti-anaphylactoid effects of Shikonin. Our results demonstrated a marked inhibitory effect of Shikonin on the allergic inflammatory responses, which might result due to the inhibition of MC activation (Fig. 1C).

The activation of MCs is critically regulated by intracellular Ca^{2+} concentrations Occhiuto et al., 2018. Our results showed that Shikonin suppressed C48/80-induced calcium mobilization in LAD2 cells (Fig. 3) and inhibited LAD2 cell degranulation. Moreover, histamine, a potent vasoactive agent released by degranulation, was dramatically decreased following Shikonin administration (Fig. 4D). Histamine is produced by decarboxylation of the amino acid histidine, a reaction catalyzed by the HDC. Therefore, induction of histidine decarboxylase replenishes the pool of MC histamine lost with degranulation occurring with exercise (Romero et al., 2017). Previous study reported that the stronger variation in histamine concentration versus HDC activity might be because of the higher spontaneous release of histamine from MCs and other types of cell, such as vascular smooth muscle and endothelial cells, under long stimulating time (Guhl et al., 2010; Tippens and Gruetter, 2004). In present study, we found that HDC activity has little variation in degranulated MC within 30 min. this indicated that synthesis of HDC enzyme needs more than one hour after histamine secreted from MC (Romero et al., 2017). Also, the present study focused on Mrgprx2-mediated MC degranulation with 30 min. We presume that HDC expression and activities need more time to regenerate histamine in MC.

Mrgprx2 has gained great attention on pseudo-allergic reactions since 2015 (McNeil et al., 2015). Recently, some active ingredients from herbs were screened to target on Mrgprx2 as antagonist for allergic-like reactions therapy (Ding et al., 2019). And some reports also investigated molecular transduction induced by Mrgprx2 Liu et al., 2017. Calcium influx is the first response of mast cell when Mrgprx2 is stimulated by certain ligands (Gaudenzio et al., 2016). Thus, we investigated of Mrgprx2-PLC-PKC-IP3 signaling which is known to influence $[Ca^{2+}]_i$ levels and MC degranulation. PLC γ 1 activation leads to PIP2 hydrolysis, which generates IP3 and DAG, thereby inducing the release of Ca^{2+} from the endoplasmic reticulum (ER) and the activation of PKC, respectively (Rivera et al., 2008). In our study, Shikonin inhibited MC activation by downregulating the phosphorylation of intracellular signaling molecules, including PLC γ 1, IP3R, and PKC (Fig. 5A). With persistent stimulation of Mrgprx2, also results in Ca^{2+} -dependent production of proinflammatory factors, chemokines synthesis and secretion from mast cells through the MAPK- and AKT-dependent pathways, such as ERK1/2, P38, and AKT (Subramanian et al., 2016b). As we known, cytokines, chemokines also play important role in eosinophil and neutrophil recruitment in allergic-related diseases, such as CU and asthma (Fujisawa et al., 2014). TNF- α seems to be the cytokine at the apex of the proinflammatory cytokine cascade, and its production contributes to the pathogenesis of both acute and chronic inflammatory diseases (Staniforth et al., 2004). Our results showed that Shikonin suppressed the secretion of cytokines such as TNF- α , and chemokines such as IL-8 (Wong and Fish, 2003), and MCP-1 both in vitro and in vivo. Moreover, Shikonin-induced inhibition of cytokine secretion could be regulated via the MAPK and PI3K/AKT signaling pathway. Western blot analysis showed that Shikonin inhibited the phosphorylation of ERK1/2 and AKT (Fig. 5C), but with no effect on P38 phosphorylation (Data not shown). Thus, deep investigation of Mrgprx2-mediated signaling pathway is very helpful to evaluate the new antagonist effects and mechanism in allergic-related diseases.

The result of molecular docking studies further supported our findings that Shikonin is a potential inhibitor of Mrgprx2 (Fig. 6B). Moreover, Shikonin was found as specific ligand on Mrgprx2 based on SPR assay. Compared with previous studies, we think that low concentration of Shikonin has good effects in attenuating pseudo-allergic reactions in vitro and in vivo. Previous study found that Shikonin can suppress C48/80 induced histamine release (Wang et al., 1995). Our study also confirmed the phenomenon and further give deep investigation of the mechanism using western blot. We found a dose-dependent repression on Mrgprx2-mediated signal pathway. Therefore, the study revealed that Mrgprx2 play central role in Shikonin inhibitory effects on pseudo-allergic reactions. Additional work can focus on therapeutic effects of Shikonin on specific allergic diseases, such as Chronic urticarial, which was observed with significantly higher expression of Mrgprx2 in skin MCs (Fujisawa et al., 2014). Moreover, the active amino acids from binding site of Mrgprx2 needs further exploration in following studies.

In conclusion, our results indicated that Shikonin potently inhibited MC activation, which was correlated to the Mrgprx2-mediated inhibition of the PLC γ -PKC-IP3 signaling pathway, suggesting that Shikonin could be a potential drug candidate for new allergic and inflammatory diseases therapies.

Declaration of Competing Interest

None.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (Grants 81872837, 81227802, and 81503321), the Fundamental Research Funds for Central University and the Foundation from the Shaanxi Administration of Traditional Chinese Medicine (Grant

15-ZY032), China Postdoctoral Science Foundation (Grant 2016 M592808), and the Natural Science Foundation of Shaanxi Province (Grants 2017JM8035).

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