



Anti-pseudo-allergy effect of isoliquiritigenin is MRGPRX2-dependent

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ABSTRACT

Mast cell (MC) is the key mediator during allergy occurs. The classical MC degranulation pathway is mediated by FcεRI aggregation and varies in strength among subjects, whereas a non-classical but analogous pseudo-allergic way was recently reported to occur via MRGPRX2. However, few therapies can directly target pseudo-allergies and related Mrgprs. Isoliquiritigenin (ISL) exerts anti-inflammatory effect in many diseases. In this study, we investigated the anti-pseudo-allergy effects of ISL and its underlying mechanism. We first examined the effect of ISL on the IgE-independent response using a PCA model, and in vitro cultured MCs. Further, we evaluated whether the anti-pseudo-allergic effect was related to Mrgprs using in vitro MRGPRX2-expressing HEK293 cells. ISL dose-dependently suppressed compound 48/80 (C48/80)-induced PCA and MC degranulation in mice. Our in vitro studies revealed that ISL reduced C48/80-induced calcium flux and suppressed degranulation in LAD2 cells. ISL dose dependently inhibited C48/80-induced MRGPRX2-expressing HEK293 cell activation. Our finding that ISL could inhibit IgE-independent allergy, via the Mrgprx2 pathway provides a new insight into pseudo-allergy and its therapy.

1. Introduction

Anaphylaxis, a severe, life-threatening allergic reaction, typically presents many different symptoms, including, angioedema, shortness of breath, stridor, and dysrhythmia. Mast cells (MCs), primary effectors in allergic reactions [1], are located primarily at the sites exposed to external environments, such as the skin, respiratory tract, and oral/gastrointestinal mucosa [2]. MCs contain secretory granules, which contain a vast array of molecules exhibiting multiple biological activities. They may have important roles in inflammatory and allergic reactions through the release of stored or *de novo*-synthesized inflammatory mediators such as histamine (HA), proteases, prostaglandins, leukotrienes, and cytokines [3–5]. HA has long been considered as an important mediator of anaphylaxis and is the first mediator released by MCs [6,7], it has multiple pathophysiologic activities, such as contraction of smooth muscles, vasodilatation, increasing vascular permeability, mucus secretion, and activation of nociceptive neuronal receptors [8].

Classically, MCs are activated through the crosslinking of antigen-specific IgE receptors (FcεRI), which results in the release of preformed and *de novo*-synthesized mediators to induce MC degranulation [9]. Although allergic MC degranulation occurs by FcεRI aggregation and varies in strength among subjects, an analogous pseudo-allergic route was recently uncovered to proceed via MRGPRX2 [10]. Ligands encompass various drugs, antimicrobial peptides, and neuropeptides such

as substance P (SP), making the receptor rather universal. These ligands stimulate MRGPRX2 directly without the necessity of an intermediary such as IgE [5].

Thus far, no therapies are available to cure allergic diseases completely. Some medicines such as anti-HA drugs, MC stabilizers, and immune suppressors can only help relieve allergic symptoms and alleviate the suffering due to anaphylaxis; moreover, these medicines have several side effects [11].

Isoliquiritigenin (ISL) is a chalcone isolated from the roots and stems of *Glycyrrhiza glabra*, *G. uralensis*, and *G. inflata* [12]. Liquiritigenin is used in common foods as an alternative medicine, and its derivative-ISL is utilized as a food additive. Other reported pharmacological properties include inhibition of cancer cell growth, and anti-inflammatory and anti-oxidative activities [13]. In 2007, Lee et al. suggested that ISL induced anti-inflammatory effects through antinephritic effects observed in high serum IgA mice could be partly attributed to the specific inhibition of platelet-derived growth factor receptor tyrosine kinase by ISL [14]. However, there are no reports on the effects of ISL in IgE-independent allergy. The aim of the present study was to determine the in vitro and in vivo effects of ISL in anti-pseudo-allergy.

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2. Materials and methods

2.1. Drugs and reagents

ISL (purity $\geq 98\%$) was obtained from Baojichenguang (Baoji, China). Compound 48/80 was purchased from Sigma-Aldrich (St. Louis, USA). Fluo-3, AM ester, and Pluronic F-127 were from Biotium (California, USA). Tyrodé's solution was prepared fresh on the day of use (6.954 g/l NaCl, 0.353 g/l KCl, 0.282 g/l CaCl_2 , 0.143 g/l MgSO_4 , 0.162 g/l KH_2PO_4 , 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). The 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.

2.2. 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 [15].

2.3. Animals

Adult male 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 ad libitum access to water, and were fed standard dry chow twice a day. The breeding environment was maintained at 20–25 °C, with relative humidity of 40% on a 12 h light/dark cycle. The mice were randomly divided into the following groups (five mice per group): control, C48/80, and the experimental group. All experiments involving equal treatments of animals were conducted by experimenters blind to the experimental conditions.

2.4. Ethics statement

This study was performed in strict accordance with the recommendations of 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.

2.5. Cell lines

Laboratory of Allergic Disease 2 (LAD2) human MCs were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). The MRGPRX2 receptor was confirmed to be present on LAD2 cells [4]. Cells were maintained in StemPro™-34 medium supplemented with 10 ml/l StemPro™ nutrient supplement, 1:100 penicillin-streptomycin, 2 mM L-glutamine, and 100 ng/ml human stem cell factor in 5% CO_2 at 37 °C. The culture medium was replaced every two weeks and cells were maintained at a density of 2×10^6 cells/ml. Human MRGPRX2-expressing HEK293 cells were constructed using HIV-1-based lentiviral vectors and cultured in DMEM.

2.6. 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 were then treated with 10 μl of ISL at different concentrations (0, 25, 50, 100 and 200 μM) for 12 h. Next, 10 μl of Cell Counting Kit solution was added to each well followed by

incubation in 37 °C for 2 h. Further, relative cell viability was assessed by detecting of absorbance at 450 nm using a microplate reader (Bio-Rad, Carlsbad, CA, USA).

For apoptosis caused by ISL, the Annexin-V-FLUOS Staining Kit from Roche Ltd (Basel, Switzerland) was used. LAD2 cells were seeded into 6-well plates at a density of 5×10^5 cells per well, and were then treated with different concentrations of ISL (0, 25, 50, 100 and 200 μM) for 12 h. All the steps were finished by strictly following the manufacturer's instructions. Apoptosis was analyzed using Accuri™ C6 Plus Flow Cytometer (Becton, Dickinson and Company, New Jersey, USA).

2.7. β -hexosaminidase assay

LAD2 cells were seeded into 96-well plates and incubated overnight at 37 °C in 5% CO_2 . The culture medium was then replaced with 100 μl per well of Tyrodé's solution containing different concentrations of ISL (25, 50 and 100 μM), and incubated for 30 min at 37 °C in 5% CO_2 . Blank and negative controls were treated with Tyrodé's solution alone under the same incubation conditions. The plates were centrifuged at $200 \times g$ for 5 min at 4 °C and the buffer was removed. 100 μl of Tyrodé's solution containing 30 $\mu\text{g}/\text{ml}$ C48/80 was added to ISL-treated cells and negative control and 100 μl of Tyrodé's solution only was added to the blank control. To analyze the total β -hexosaminidase content, cells were lysed with 0.1% Triton X-100 in Tyrodé's solution. β -hexosaminidase released into the supernatants and 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 the stop buffer (0.1 M sodium carbonate/sodium bicarbonate, pH 11). 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.

2.8. HA release assay

LAD2 cells (1×10^6 cells per well) were incubated in a 96-well tissue culture plate overnight at 37 °C with 5% CO_2 . Following incubation, the culture medium was removed and 100 μl of ISL at the indicated concentrations (12.5, 25 and 50 μM) was prepared by adding tyrodé's solution. Only Tyrodé's solution was added in the blank and negative control. Cells were incubated for 30 min at 37 °C with 5% CO_2 . The 96-well plate was centrifuged at $200 \times g$ for 5 min at 4 °C and the buffer was discarded. Hundred microliter of Tyrodé's solution containing 30 μl of C48/80 was added in the ISL and negative control groups, 100 μl Tyrodé's solution was used as the blank control. The HA release assay was performed using LC-ESI-MS/MS method. HA and formic acid (mass spectrometry grade) were purchased from Sigma, HA $\cdot$ 2HCl (A, A, B, B-D4, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA), and HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, USA). In the applied LC-ESI-MS/MS method, an LCMS 8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used. HA was evaluated on the system with an HILIC column (Venusil HILIC, 2.1 mm $\times$ 150 mm, 3 μm , Agela Technologies, Tianjin, China), and isocratic elution was performed 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.

2.9. Chemokine release assay

LAD2 cells (1×10^6 per well) were incubated overnight in a 96-well tissue culture plate at 37 °C. After the culture medium was removed, 100 μl of different concentrations of ISL (12.5, 25, 50 μM), prepared in Tyrodé's solution and containing 30 $\mu\text{g}/\text{ml}$ compound 48/80, was added to each well and incubated for 6 h at 37 °C. TNF- α , MCP-1, and

IL-8 ELISA Kits were purchased from BIOTECH CO., LTD. (Beijing, China) to measure the levels of TNF- α , MCP-1, and IL-8 levels, respectively, in the supernatants according to provided the manufacturer's instructions.

2.10. Intracellular Ca^{2+} mobilization assay

The incubation buffer consisted of 0.5 μl of Fluo-3, 2 μl of Pluronic F-127, and 997.5 μl of calcium imaging buffer (CIB; 125 mM NaCl, 3 mM KCl, 2.5 mM CaCl_2 , 0.6 mM MgCl_2 , 10 mM HEPES, 20 mM glucose, 1.2 mM NaHCO_3 , 20 mM sucrose; pH 7.4). Different concentrations of ISL (12.5, 25 and 50 μM) were prepared in the incubation buffer, an incubation buffer without ISL was used as the negative control.

LAD2 cells were washed twice with CIB, followed by the addition of the required incubation buffer. After 30 min of incubation, cells were washed twice with CIB and plated into a 96-well plate for calcium imaging.

For determining the MC response, LAD2 cells were prepared with the same method. Next, the drugs were added into the well to determine the number of cells and increase in fluorescence.

MRGPRX2-HEK293 cells or NC-HEK293 cells were plated at 1×10^4 cells per well in a 96-well tissue culture plate and incubated overnight at 37 °C. Cells were then washed twice with CIB, followed by the addition of the required incubation buffer. After 30 min of incubation at 37 °C, cells were again washed twice with CIB and subjected to imaging. For calcium imaging, one photo per second was captured under blue light (200 $\times$ magnification). Cells were identified to be responding if the concentration of $[\text{Ca}^{2+}]$ increased by at least 50% after injecting the substances.

2.11. EC_{50} assay

Human MRGPRX2-HEK293 cells (2×10^4 per well) were incubated overnight in a 96-well plate at 37 °C. FLIPR Calcium 5 Assay Kit was purchased from Molecular Devices, LLC (California, USA). EC_{50} value was determined using FlexStation 3. Indicated concentrations of ISL (12.5, 25 and 50 μM) were prepared by component A and C 48/80 was used as an agonist.

2.12. Hind paw swelling and extravasation

Adult male mice weighing 18–22 g, were anaesthetized with an intraperitoneal injection of 0.08 ml 10% chloral hydrate. Fifteen minutes after the induction of anesthesia, each mouse was intravenously (i.v.) injected 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. After five minutes, 5 μl of the test drug and 30 $\mu\text{g}/\text{ml}$ of C48/80 in saline were injected into the left paw, saline only was injected into the right paw as the blank control. After 15 min, paw thickness was measured again and recorded. Mice were sacrificed by decapitation, and a photo of each paw was captured. Paw tissues were collected, dried for 24 h at 50 °C, and weighed. Evans blue was extracted by 24 h of incubation in 500 μl acetone-saline (7:3) at 37 °C. Tissues were cut into pieces, placed for 10 min in an ultrasonic machine, and centrifuged for 20 min at 3000 rpm. The supernatant was equally distributed in 200 μl aliquots and placed into 96 well cell culture plates, and the OD was determined at 620 nm using a spectrophotometer. For studies using drugs, mice were injected with 5 μl of 30 $\mu\text{g}/\text{ml}$ C48/80 as the negative control.

For the HA release assay, a similar procedure was performed. Briefly, anaesthetized mice were injected with 5 μl of the test drug with 30 $\mu\text{g}/\text{ml}$ C48/80 into the left paw and saline into the right paw as the blank control. Mice were injected with 5 μl of 30 $\mu\text{g}/\text{ml}$ solution of compound 48/80 as the negative control. Fifteen minutes later, paws were collected weighed, cut into pieces and placed in EP tubes with

400 μl of ultrapure water. The supernatant, obtained by centrifugation at 5000 rpm for 20 min, was used in the HA release assay by LC-ESI-MS/MS method.

2.13. Histological analysis

Anaesthetized mice were injected with 5 μl of the test drug and 30 $\mu\text{g}/\text{ml}$ C48/80 saline in the left paw; saline and C48/80 were used as blank 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 with the 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 three times with PBS, and a drop of Fluoro-mount G (Southern Biotech, AL, U.S.A) was added. Images were captured immediately using a confocal scanning laser microscope (Nikon, Tokyo, Japan).

2.14. Cell membrane chromatography

The harvested MRGPRX2-HEK293 and NC-HEK293 cells (7×10^6) at 80–90% confluency were washed three times with 5 mM PBS by centrifugation at $3000 \times g$ for 10 min, and the pellet was re-suspended in 50 mM Tris-HCl (pH 7.4), followed by ultrasonication for 30 min. The resulting homogenate was clarified by centrifugation at $1000 \times g$ for 10 min, and the supernatant was centrifuged at $12\,000 \times g$ for 10 min. The precipitate was then suspended in 5 mM PBS [16]. Briefly, the cell membrane stationary phase (CMSP) was prepared by adsorption of the cell membrane suspension (5 ml) on activated silica (0.05 g) under vacuum with gentle agitation. The CMSP was placed overnight and then washed five times with 5 mM PBS. Finally, the mixture obtained was packed into a column (10 $\times$ 2.0 mm I.D.) using a wet packing method (10 MPa, 5 min). All procedures were performed at 4 °C. After MRGPRX2-HEK293/CMC columns were prepared, they were attached to the LC-2010AHT liquid chromatograph, and the conditions were as follows: mobile phase, 1 mM PBS; flow rate, 0.2 ml/min; and UV detection, 370 nm.

2.15. Statistical analysis

Data were expressed as mean $\pm$ S.E.M. Independent sample variance analysis was used to determine significance in statistical comparisons using SPSS. Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

3. Results

3.1. ISL is a potential ligand to MRGPRX2 receptor

Molecular docking has a wide range of uses and applications in drug discovery, including finding potential ligands by virtual drug screening. Results of molecular docking experiments demonstrated that ISL binds to MRGPRX2 receptor protein through hydrogen-bond interactions. The sphere space field model (Fig. 1A) showed that ISL has a good fit within the active cavity of MRGPRX2. Further analysis of the interaction between ISL and MRGPRX2 (Fig. 1B) demonstrated that three hydroxyls of ISL formed three hydrogen bonds with PHE147, CYS155, and ASP159, respectively (bond lengths were 1.95 Å, 2.22 Å, and 1.93 Å, respectively).

Moreover, the results of cell count and apoptosis all showed that 12 h treatment with different concentrations of ISL (12.5, 25, and 50 μM) had no cytotoxic effect, whereas treatment with 100 and 200 μM of ISL exhibited a slight negative effect on LAD2 cells (Fig. 1C and D).

We observed no increase in intracellular Ca^{2+} ion concentrations in

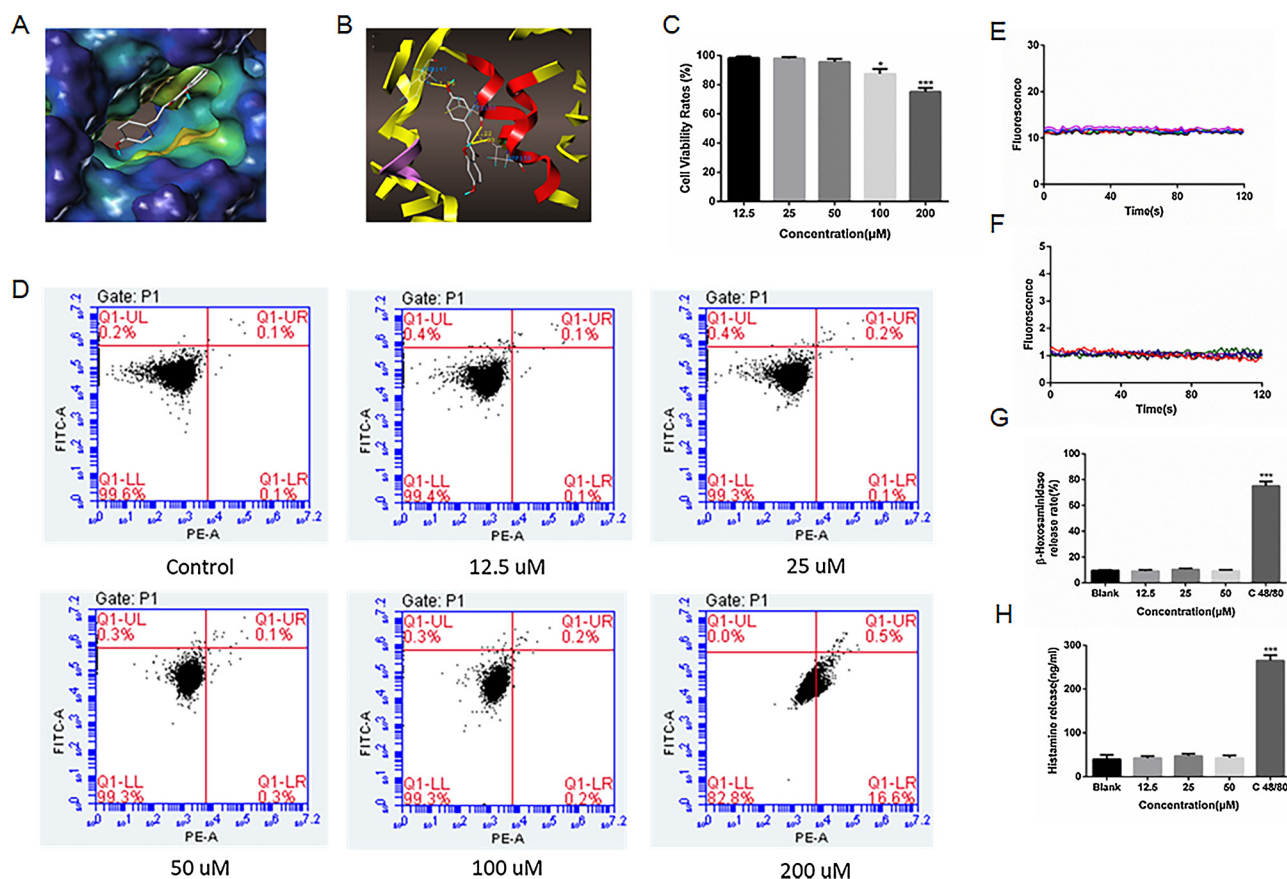


Fig. 1. ISL as a potential MRGPRX2 antagonist. (Experiments were repeated a minimum of three times. Data are presented as mean $\pm$ S.E.M. and were analyzed two-tailed unpaired Student's *t*-test.) (A–B) ISL binds to MRGPRX2 protein receptor as observed by the molecular docking assay. Three hydroxyls of ISL formed three hydrogen bonds with PHE147, CYS155, and ASP159, and the bond lengths were 1.95 Å, 2.22 Å, and 1.93 Å, respectively. (C) ISL showed no cytotoxic effect on LAD2 cells. (D) ISL did not induce LAD2 apoptosis. (E–F) Fifty micromolar ISL could not activate LAD2 cells or MRGPRX2-expressing HEK-293 cells to increase the intracellular calcium ion (Ca^{2+}) concentration. (G–H) ISL failed to induce LAD2 cell degranulation and release of β -hexosaminidase and histamine. C48/80 (30 $\mu\text{g}/\text{ml}$) was used as the positive control, and Tyrode's solution was used as the blank control.

LAD2 or MRGPRX2-expressing HEK293 cells after ISL treatment, suggesting that ISL does not affect the activation of LAD2 and MRGPRX2-expressing HEK293 cells (Fig. 1E and F). Moreover, ISL treatment did not induce LAD2 cell degranulation in vitro and had no detectable effect on the release of β -hexosaminidase and HA from LAD2 cells (Fig. 1G and H), indicating that ISL may exhibit an inhibitory effect on the degranulation of LAD2 cells.

3.2. ISL reduced C48/80-induced calcium flux and degranulation in LAD2 cells

MC degranulation and HA production is Ca^{2+} -dependent. C48/80 induces an increase in MC Ca^{2+} flux and results in MC activation. Therefore, to determine the effect of ISL on calcium flux and activation of MC, LAD2 cells were pretreated with ISL for 30 min and then loaded with Fluo-3 AM. Indeed, after ISL treatment, a dose-dependent decrease in the calcium flux was observed in LAD2 cells (Fig. 2A and B) and the response of LAD2 cells significantly decreased suggesting that ISL inhibited C48/80-induced MC activation.

In addition, when the concentration of ISL increased, the response of LAD2 cells reduced because ISL probably exhibited competitive inhibition with C48/80 on the MRGPRX2 receptor (Fig. 2C).

Degranulation is another hallmark of activated MCs. Therefore, we measured the release of β -hexosaminidase and HA, which are prestored mediators, to determine whether ISL treatment inhibits C48/80-induced MC degranulation. ISL assay showed that addition of C48/80 induced LAD2 cell degranulation, whereas treatment with ISL dose dependently

inhibited degranulation of LAD2 cells (Fig. 3A). This inhibition of degranulation by ISL was also supported by the finding that HA secretion was reduced in ISL-treated LAD2 cells (Fig. 3B). These results are consistent with the observations that ISL treatment inhibited MC degranulation.

In addition, ISL dose dependently inhibited the release of $\text{TNF-}\alpha$, MCP-1, and IL-8 cytokines (Fig. 3C–E) by LAD2 cells.

3.3. ISL inhibited C48/80-induced local anaphylaxis

To investigate the anti-pseudo-allergy effect of ISL, a mouse model of C48/80-induced skin inflammation, previously shown to be IgE-independent, was used. Injection of ISL and C48/80 mixture into mouse hind paws dose-dependently suppressed local anaphylaxis. The degree of swelling and Evans blue exudation of the mice paw were reduced (Fig. 4A). Subsequently, we analyzed HA release and demonstrated that ISL reduced HA release in mice paw (Fig. 4B). The inhibitory effect was observed as a decrease in hemangiectasis caused by HA (Fig. 4C). We then performed avidin staining of the related paw skin sections and observed a significant decrease in the percentage of degranulated MC after ISL treatment, suggesting that ISL antagonized MCs in vivo to their degranulation (Fig. 4D).

3.4. Anti-pseudo-allergy effect of ISL is related to MRGPRX2 expression

Studies suggest that C48/80-induced MC activation is related to MRGPRX2, the target of many small molecule drugs associated with

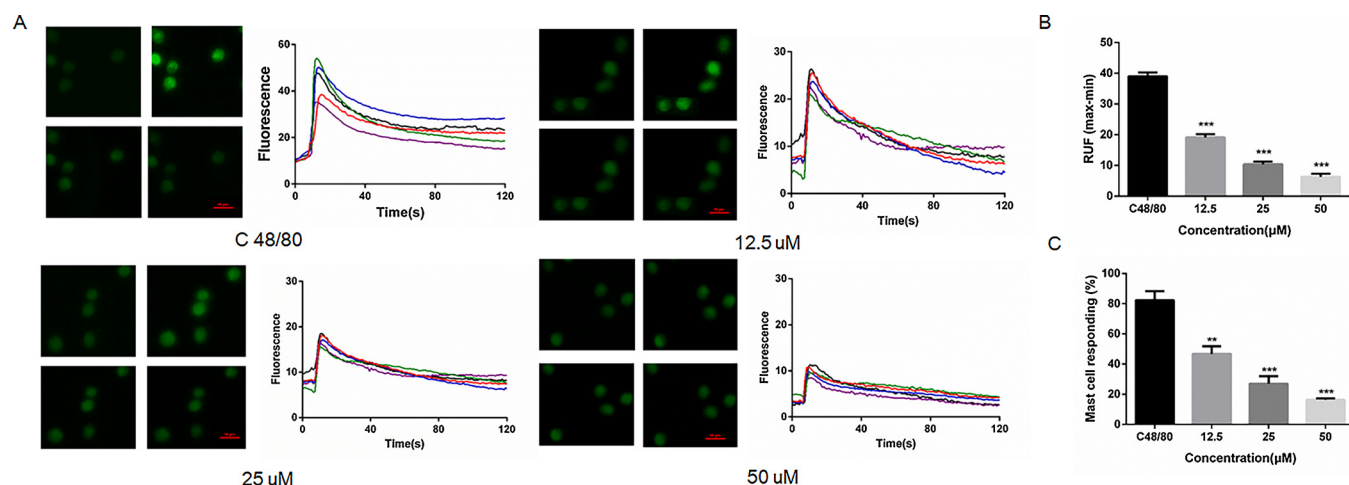


Fig. 2. ISL reduced C48/80-induced calcium (Ca^{2+}) flux in LAD2 cells. (Data are expressed as mean $\pm$ S.E.M from at least three independent experiments. Analysis was performed using two-tailed unpaired Student's *t*-test.) (A) (A) C48/80 (30 $\mu\text{g}/\text{ml}$) was used as an agonist for LAD2 cells and as the negative control. The concentration of ISL was set as 12.5, 25, and 50 μM . ISL inhibited C48/80-induced increase in intracellular Ca^{2+} concentration in LAD2 cells in a dose-dependent manner. (B) ISL reduced the fluorescence intensity of C48/80-treated LAD2 cells in a dose-dependent manner. (C) Response of LAD2 cells to ISL treatment following C48/80 activation was assessed by Fluo-3 imaging.

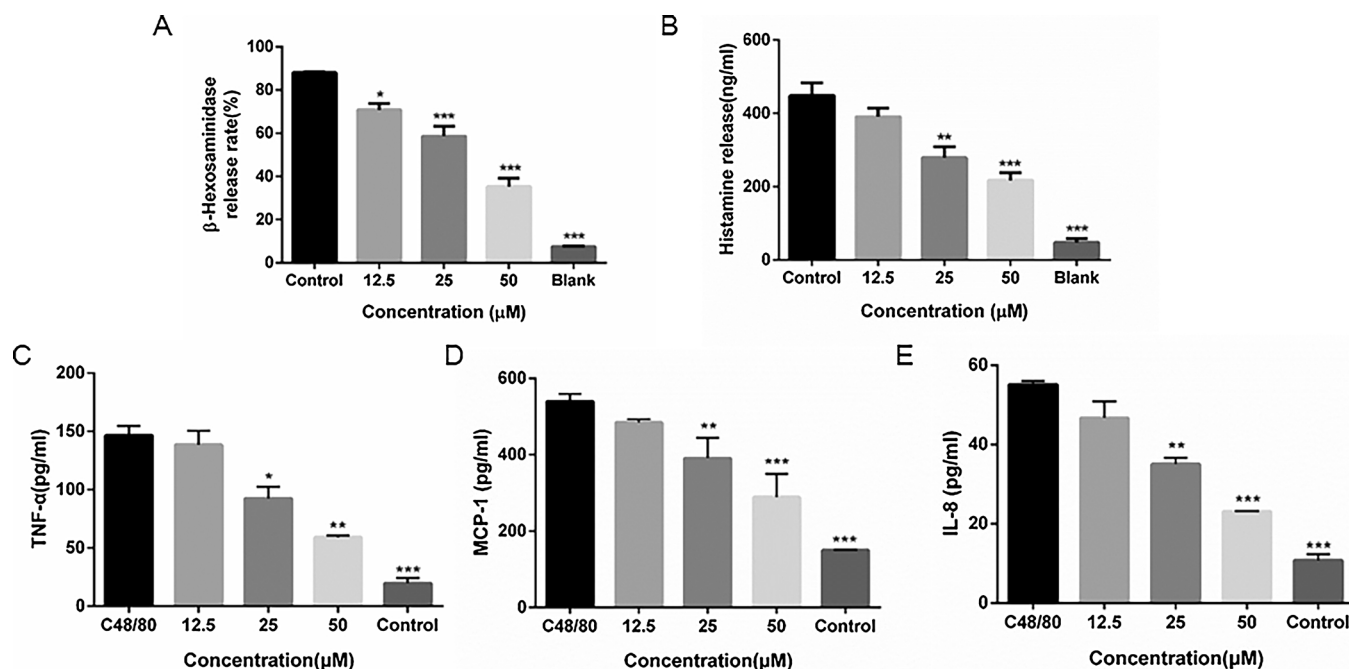


Fig. 3. ISL inhibited C48/80-induced degranulation and cytokine release in LAD2 cells. C48/80 (30 $\mu\text{g}/\text{ml}$) was used as an agonist for LAD2 cells and as the negative control. Tyrode's solution was used as the blank control. Different concentrations of ISL (12.5, 25, and 50 μM) were used to inhibit the degranulation caused by C48/80. Data are expressed as mean $\pm$ S.E.M from at least three independent experiments. Analysis was performed using two-tailed unpaired Student's *t*-test. (A-B) ISL inhibited C48/80-induced degranulation. ISL reduced release of β -hexosaminidase and histamine. (C-E) ISL inhibited C48/80-induced release of cytokines, such as TNF- α , MCP-1, and IL-8 in LAD2 cells.

systemic pseudo-allergic or anaphylactoid reactions in humans. C48/80 could not increase Ca^{2+} flux in the NC-HEK293 cells because they did not express MRGPRX2 (Fig. 5A). Therefore, MRGPRX2-expressing HEK-293 cells, pre-treated with different concentrations of ISL before C48/80 activation were used, and these cells showed dose-dependent inhibition of cell activation (Fig. 5B). In addition, MRGPRX2-expressing HEK-293 cells, and EC_{50} value of C48/80 to activate the MRGPRX2 receptor increased in a dose dependent manner with ISL (Fig. 5C), indicating that the anti-pseudo-allergy effect of ISL was related to MRGPRX2 expression.

Furthermore, cell membrane chromatography (CMC) is a type of biological affinity chromatography to demonstrate receptor-ligand

binding. Morphine, which is an agonist to MRGPRX2 receptor [15], was used in this study. Both ISL and morphine bound in MRGPRX2-HEK293 CMC, but not in NC-HEK293 CMC (Fig. 5D and E), indicating that ISL and morphine were the ligands of the MRGPRX2 receptor. However, molecular docking of morphine with MRGPRX2 demonstrated that morphine formed one hydrogen bond with ASN60, and the bond length was 2.08 Å (Fig. 5F). The spatial position between ISL and morphine with the active MRGPRX2 cavity was different. Agonists and antagonists were combined in different binding sites, although they were both related to MRGPRX2. However, ISL also positively inhibited anti-pseudo-allergy induced by morphine. (Fig. 5G). Therefore, we considered that the anti-pseudo-allergy effect of ISL was related to

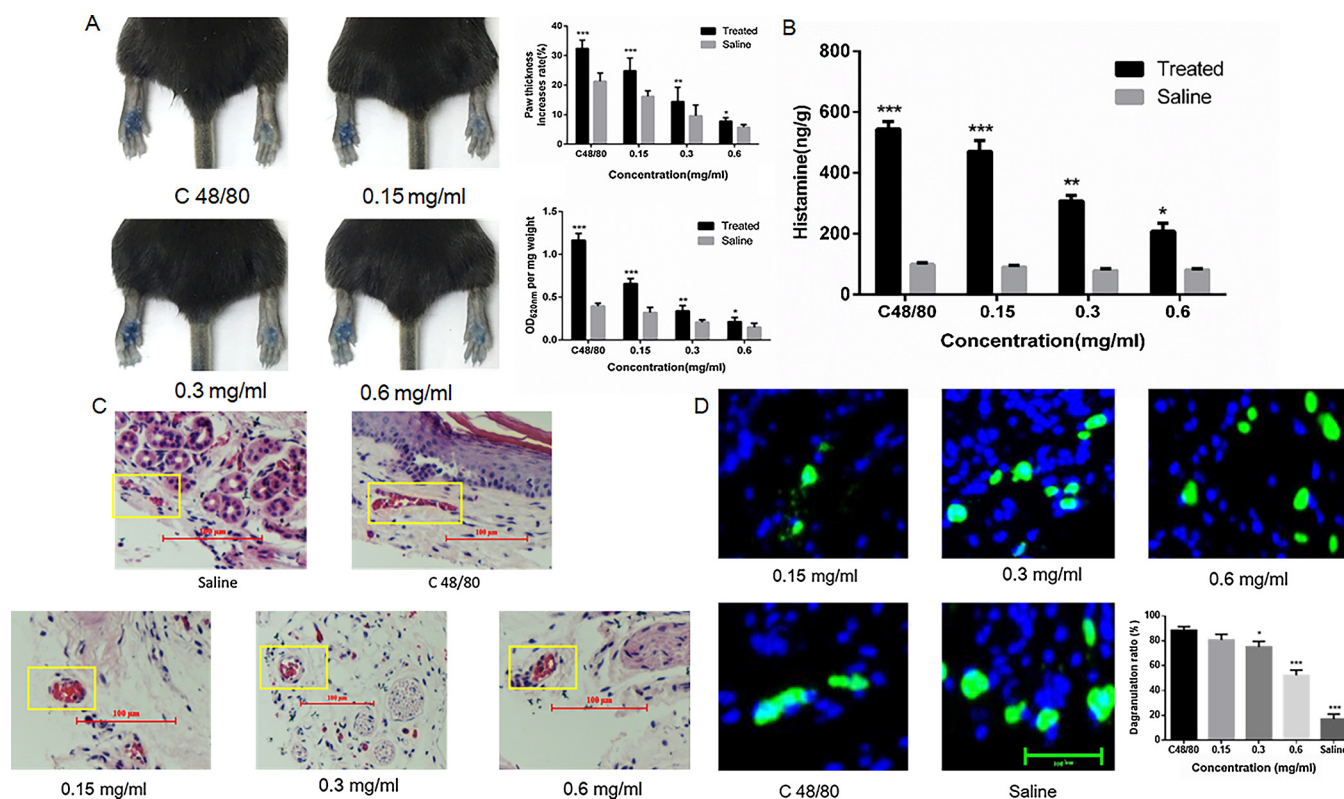


Fig. 4. ISL inhibited C48/80-induced local anaphylaxis by inhibiting mast cells degranulation. Treat group indicates that 30 μ g/ml C48/80 with different ISL concentrations (0, 0.15, 0.3, 0.6 mg/ml) were injected into the left paw. Saline was simultaneously injected into the right paw as the blank control. All experiments were repeated at least three times ($n = 5$). Data are expressed as mean $\pm$ S.E.M. and analyzed using two-tailed unpaired student's *t*-test. (A) ISL reduced the degree of swelling and Evans blue exudation in mice hind paws induced by C48/80. (B) ISL reduced histamine release in mice hind paws. (C) ISL inhibited C48/80-induced hemangiectasis as demonstrated by H&E staining of skin tissue sections. (D) ISL inhibited mast cells degranulation (avidin staining).

MRGPRX2.

4. Discussion

ISL is an active component in several plants [11]. Although ISL suppression of inflammatory factors has been described previously [17], its anti-allergy effect remains controversial. In this study, we showed that the anti-allergy effects of ISL were mediated by the inhibition of MC activation both in vitro and in vivo.

MCs are the key mediators during allergy. There are two main pathways for MC activation: IgE-dependent and IgE-independent. In addition to the high-affinity IgE receptor (Fc ϵ RI), which is IgE-dependent, MCs express numerous G protein-coupled receptors (GPCRs), which are the largest group of membrane receptor proteins and the most common targets of drug therapy. Antimicrobial host defense peptides, neuropeptides, major basic protein, eosinophil peroxidase, and many US Food and Drug Administration-approved peptidergic drugs activate human MC through a novel GPCR, known as MRGPRX2 [2].

Because MCs are acute effector cells in allergy we used LAD2, a human MC line, to study the inhibition effect of MC activation by ISL. Cytosolic Ca²⁺ is a universal and highly versatile signal [18], and in MCs, Ca²⁺ activates a complex cascade of signals, which leads to degranulation of pre-stored mediators, de novo synthesized lipid mediators, pro-inflammatory cytokines, and chemokines [19,20]. We assessed the toxic effect of ISL on LAD2 cells. Results of in vitro experiments suggested that ISL pretreatment dose dependently inhibited C48/80-induced activation and reduced degranulation of LAD2 cells, suggesting that ISL inhibited C48/80-induced granule release in MC.

As tissue-resident cells, MCs are strategically situated at host-environment interfaces, such as the skin, respiratory tract, and

gastrointestinal tract, and can readily respond to immunogenic stimuli [21]. We performed a skin allergic test to evaluate the anti-pseudo-allergy effect of ISL using skin samples containing MCs. Mast cells release several substances such as HA, inflammation factors, 5-hydroxytryptamine (5-HT), which induce allergy. In the C48/80 group, HA release in the skin resulted in an increase in blood vessel permeability (Fig. 4C), whereas ISL inhibited blood vessel permeability, which is likely related to MC stabilization (Fig. 4D).

Because MRGPRX2 is an acute factor in IgE-independent allergy, we used MRGPRX2-transfected HEK293 cells to determine the effect of ISL on these cells. As shown in the results, when pretreated with ISL, EC₅₀ value of C48/80-induced MRGPRX2 HEK cell activation increased dose dependently (Fig. 5), thus, further supporting the result that anti-pseudo-allergy effect of ISL is dependent on MRGPRX2. Molecular docking, an important method for drug virtual screening, is a simple and convenient method to determine the protein ligand. However, because the ligand may either be an agonist or antagonist, functional experiments are necessary to verify the effect of the compound on the receptor.

In our study, molecular docking results suggested that ISL is a potential MRGPRX2 ligand and that the inhibitory effect of ISL on MC activation is dependent on MRGPRX2 (Fig. 1A and B), the main target of pseudo-allergy. However, unfortunately, competitive displacement was not applicable in this study because agonists and antagonists for a receptor were combined at different binding sites in most cases. Furthermore, ISL inhibited C48/80- and morphine-induced degranulation, which are MRGPRX2 agonists. Our findings suggest that the anti-pseudo-allergic effect of ISL is related to MRGPRX2 and, hence, evaluating the use of ISL in medical care may be important.

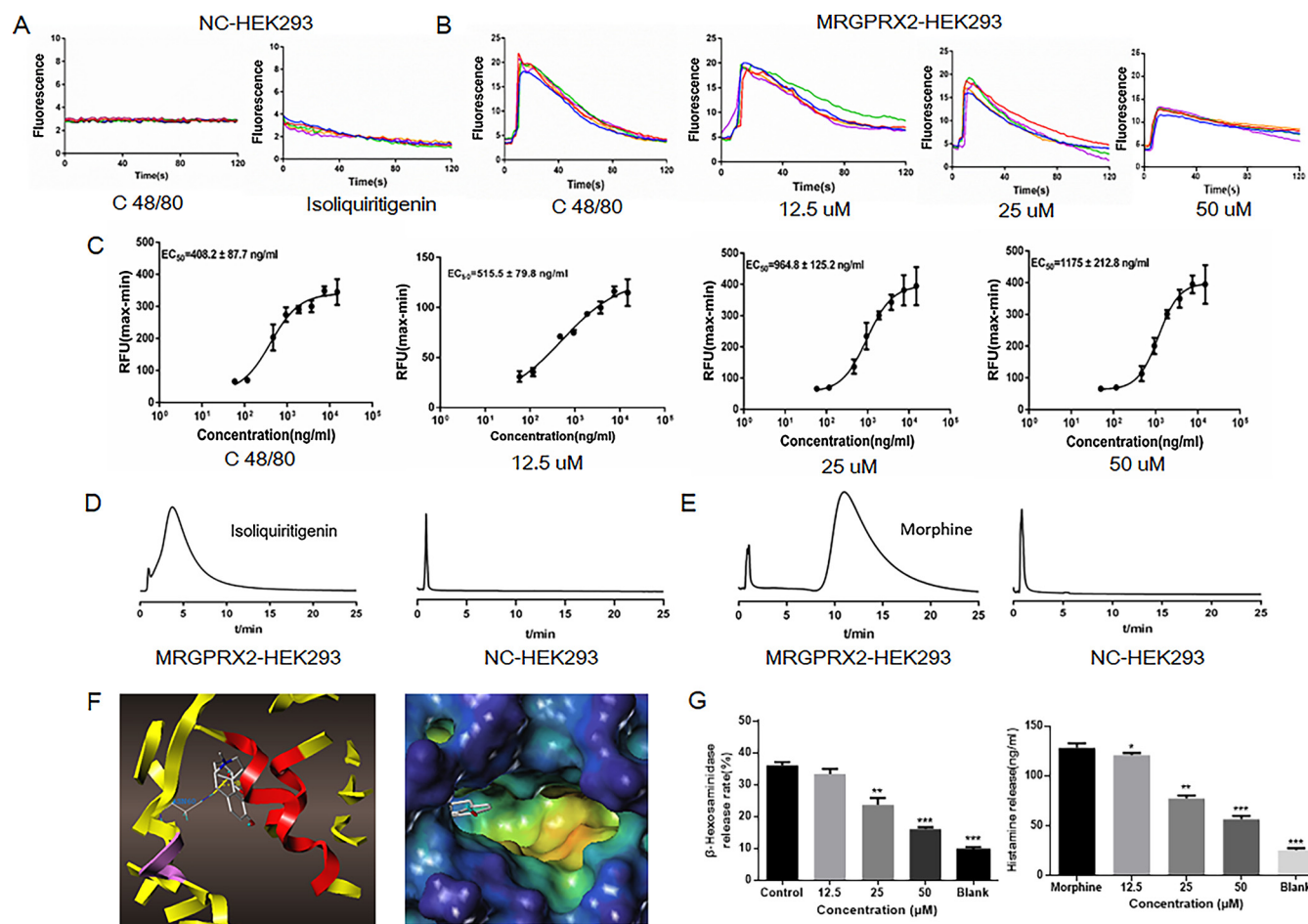


Fig. 5. ISL inhibited C48/80-induced pseudo-allergy via MRGPRX2. Experiments were repeated more than three times. Data are presented as mean $\pm$ S.E.M. and were analyzed by two-tailed unpaired Student's *t*-test. (A) Both C48/80 and ISL did not induce intracellular calcium ion (Ca^{2+}) concentration in HEK-293 cells. (B) ISL inhibited the increase in intracellular Ca^{2+} concentration in MRGPRX2-expressing HEK-293 cells caused by C48/80 in a dose-dependently manner. (C) The EC_{50} value of C48/80 to active MRGPRX2 receptor was increased with a dose manner of ISL. ISL increased the EC_{50} value of C48/80 to activate the MRGPRX2 receptor in a dose-dependent manner. (D) Chromatograms of ISL analyzed by MRGPRX2-HEK293/cell membrane chromatography (upper panel) and HEK293/cell membrane chromatography (lower panel). (E) Chromatograms of morphine analyzed by MRGPRX2-HEK293/cell membrane chromatography (upper panel) and HEK293/cell membrane chromatography (lower panel). (F) A model of the bonding pattern of morphine with MRGPRX2; morphine formed one hydrogen bond with ASN60, and the bond length was 2.08 Å. (G) ISL inhibited C48/80-induced degranulation in LAD2 cells and reduced the release of β -hexosaminidase and histamine.

Conflict of interest

None.

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