International Immunopharmacology 71 (2019) 22–31

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Isosalvianolic acid C-induced pseudo-allergic reactions via the mast cell specific receptor MRGPRX2

Yuanyuan Lin[a,b,c], Jue Wang[a,b], Yajing Hou[a,b], Jia Fu[a,b], Di Wei[a,b], Qianqian Jia[a,b], Yanni Lv[a,b,c], Cheng Wang[a,b], Shengli Han[a,b], Langchong He[a,b,*]

[a] School of Pharmacy, Xi'an Jiaotong University, 76# Yanta West Road, Xi'an 710061, China.
[b] National-provincial Joint Engineering Research Center for National Vascular Medicine Screening & Analysis, Xi'an 710061, China
[c] School of Pharmacy and Department of Chemistry, University of Wisconsin-Madison, Madison, WI, USA

ARTICLE INFO

Keywords:
Isosalvianolic acid C
Mast cell
MRGPRX2
MrgprB2
Pseudo-allergic

ABSTRACT

Pseudo-allergic reactions occurred in patients administered drugs for the first time, seriously threaten man's survival. Due to the frequent reports of pseudo-allergic reactions to Danshen injection, in our previous study, isosalvianolic acid C in Danshen injection was found to trigger off mast cell degranulation. However, the direct involvement and the mechanisms underlying pseudo-allergic reactions have not been elucidated. In this study, the pseudo-allergic reactions induced by isosalvianolic acid C were confirmed by an ear swelling assay, a hindpaw swelling and extravasation assay in vivo and mast cell degranulation assays in vitro. We also evaluated whether the pseudo-allergic effect is related to MRGPRX2, Isosalvianolic acid C induced Ca^{2+} mobilization was verified as MRGPRX2-related by Ca^{2+} imaging using mouse peritoneal mast cells (both wild-type and MrgprB2 knockout mice), MRGPRX2-expressing HEK293 and MrgprB2-expressing HEK293 cells. MRGPRX2-related pseudo-allergic reactions induced by Isosalvianolic acid C were further confirmed by MrgprB2 knockout mice and MRGPRX2 knockdown mast cells both exhibited reduced isosalvianolic acid C-induced pseudo-allergic effects. Furthermore, both the frontal analysis and molecular docking assays showed that isosalvianolic acid C has a considerable affinity with MRGPRX2. Based on the above experiments, the western blot analyses were conducted, the results indicated that isosalvianolic acid C induced Ca^{2+} mobilization and degranulation via the activation of PLC-γ and IP3R, and releasing chemokines via the activation of PLC-γ, PKC and P38. This study should alarm many clinicians that medicines containing isosalvianolic acid C might induce pseudo-allergic reactions, and it may provide guidance on safe dosage of these medicines in the process of production and use.

1. Introduction

The population-based studies estimate the incidence of pseudo-allergic reactions in western countries in the range of roughly 4–50 per 100, 000 person-years, and the pseudo-allergic reactions is very terrible that can be rapid in onset and occasionally fatal [1]. Mast cells (MCs) are the key effectors in pseudo-allergic reactions, there are two methods for MC activation (IgE-dependent and IgE-independent way). For the IgE-dependent pathway, MCs are activated through the crosslinking of IgE and IgE high affinity receptor (FcεRI) [2,3], which have been well-studied in recent years. In addition, researchers have found that human MCs express MRGPRX2, which is related to MC activation through an IgE-independent pathway, it also confirmed that Mrgprb2 in mice is orthologous to MRGPRX2 [4]. MRGPRX2 could be activated by a variety of basic compounds, peptides and amines, such as substance P, mastoparan, and compound 48/80. Further, opioid compounds, quinolone antibiotics, neuromuscular relaxants, antifungal drugs, aminoglycosides and sulfonamides have been found can activate MCs through MRGPRX2, and induce mast cell release of histamine, various inflammatory and immunomodulatory substances [5–7].

Isosalvianolic acid C (Isosal C) was found in the aqueous extract of *Salvia miltiorrhiza* in 1992 [8], one of the most commonly used traditional Chinese medicine clinically [9–11]. Isosal C plays an important role in anti-LDL-peroxidative activity [12], and was considered as a potential component for treatment of Alzheimer's diseases [13]. The traditional Chinese medicine injections (TCMIs) containing *Salvia miltiorrhiza* is one of *Salvia miltiorrhiza* preparation containing a large amount of Isosal C, have received considerable attention due to the frequent incidence of adverse drug reactions including pseudo-allergic reactions [14–16]. So, in previously study, we screened the Danshen

* Corresponding author at: School of Pharmacy, Xi'an Jiaotong University, Xi'an 710061, China.
E-mail address: helc@mail.xjtu.edu.cn (L. He).

https://doi.org/10.1016/j.intimp.2019.03.013
Received 7 November 2018; Received in revised form 13 February 2019; Accepted 6 March 2019
Available online 12 March 2019
1567-5769/

injection and found that Isosal C could cause the mast cell degranulation [17]. However, the further study and the specific mechanisms by which Isosal C activates mast cells and triggers pseudo-allergic reactions is still uncertainty.

In this study, we investigated whether Isosal C induced pseudo-allergic reaction through MRGPRX2, and the mechanism of Isosal C-induced pseudo-allergic reactions was also clarified. These findings would provide a reference for drugs containing Isosal C in the process of production and clinical use.

2. Experimental procedures

2.1. Drugs and reagents

Compound 48/80, Evans blue and *p*-nitrophenyl *N*-acetyl-β-D-glucosamide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Isosal C (purity ≥ 98%) was obtained from Wuhan ChemFaces Biotechnology Co., Ltd. (Wuhan, China). Saline was purchased from Shandong Qilu Pharmaceutical Co., Ltd. (Shandong, China). A Human MCP-1 ELISA Kit was obtained from ExCell Biology, Inc. (Shanghai, China). Fluo-3 AM was purchased from ThermoFisher Scientific (Waltham, MA). Pluronic F-127 was purchased from Biotium (Fremont, CA). The *p*-nitrophenyl *N*-acetyl-β-D-glucosamide and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Histamine-d4 (purity ≥ 98%) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA). HPLC-grade methanol and acetonitrile were purchased from Merck KGaA (Darmstadt, Germany). All aqueous solutions were prepared using ultrapure water produced via a MK-459 Millipore Milli-Q Plus ultra-pure water system (Darmstadt, Germany).

2.2. Mouse models

C57BL/6 (WT) mice were provided by the Experimental Animal Center at Xi'an Jiaotong University (Xi'an, China). MUT mice on a C57BL/6 background were kindly gifted by Professor Xinzhong Dong from Johns Hopkins University (MD, USA) [4], and C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). The mice were housed at the Experimental Animal Center of Xi'an Jiaotong University and were provided food and water ad libitum. All experiments employed adult mice weighing 25–30 g, and experiments requiring identical treatment administration in animals were conducted by investigators blinded to the conditions.

2.3. Ethics statement

The study was implemented strictly according to the recommendations stated in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocols for the use of mice were ratified by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (permit number: XJTU 2011-0045).

2.4. Cell lines

LAD2 human MCs were kindly provided by A. Kirshenbaum and D. Metcalfe from NIH (MD, USA) and were maintained in StemPro-34 medium supplemented with StemPro nutrient supplement, penicillin/streptomycin (1:100) (HyClone, UT), 100 ng/mL human stem cell factor and 2 mM L-glutamine in a 37 °C incubator at 5% CO_2. Culture medium was hemi-depleted and replaced every week to maintain the cells at a density of 2×10^6 cells/mL. HEK293-MRGPRX2 and HEK293-MrgprB2 cells were also provided by Professor Xinzhong Dong and cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Clontech, Mountain View, CA, USA) and penicillin/streptomycin (1:100) [4].

2.5. Ear swelling

Young adult mice were injected in the tail vein at a dose of 0.21, 0.52, or 1.04 mg/kg Isosal C in 4% Evans blue (0.2 mL). One hour (1 h) later, mice were sacrificed, and the ears were removed to measure dye extravasation. The dye was extracted overnight from the ear in acetone/physiological saline (7:3) solution. After ultrasonication for 30 min and centrifugation at 10000 × *g* for 20 min, 150 μL of supernatant was taken to measure the absorbance at 620 nm.

2.6. Hindpaw swelling and extravasation assay

Mice were anesthetized by an intraperitoneal injection of 70 mg/kg pentobarbital sodium. Fifteen minutes later, every mouse was intravenously (i.v.) injected with 50 μL of 4% Evans blue in saline, and paw thickness was measured by a Vernier caliper prior to the injection of any test substances. After 5 min, Isosal C or 30 μg/mL C48/80 was injected into one paw, and saline was injected into the other paw as a negative control. Paw thickness was then measured again after 15 min. The mice were sacrificed, and paw tissues were collected, dried at 50 °C and weighed separately. Evans blue dye in the paw tissues was extracted by adding 500 μL of a mixture of acetone/saline (7:3) to each tissue sample and incubated at 37 °C for 12 h. Tissues were then minced, disrupted by ultrasonication for 10 min and centrifuged at 3000 rpm for 20 min. The supernatant was seeded equally into 96-well plates (200 μL/well), and the OD value at 620 nm was measured by a microplate reader.

2.7. Peritoneal MC purification assay

The mice were sacrificed by CO_2 inhalation. A total of 12 mL of ice-cold MC dissociation media (MCDM; HBSS with 3% FBS and 10 mM HEPES, pH 7.2) was used to make two to three sequential peritoneal lavages, which were combined, and cells were centrifuged at 200 × *g* at 4 °C for 5 min. MCDM (2 mL) was used to resuspend the cells from each mouse, and the cells were layered over 4 mL of an isotonic 70% Percoll suspension (2.8 mL of Percoll, 320 μL of 10 × HBSS, 40 μL of 1 M HEPES, and 830 μL of MCDM) and centrifuged at 500 × *g* at 4 °C for 20 min. MCs were recovered in the pellet, and the purity was assayed by morphology and Toluidine blue staining (> 95%). MCs were resuspended at concentrations of 5×10^5–1×10^6 cells/mL in DMEM supplemented with 10% FBS, 25 ng/mL recombinant mouse stem cell factor (SCF) and penicillin/streptomycin (1:100), and cells were then seeded into 96-well plates.

2.8. Intracellular Ca^{2+} mobilization assay

All drugs were diluted to the required concentration using 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 $NaHCO_3$, and 20 mM sucrose; brought to pH 7.4 using NaOH). The incubation buffer contained 4 μM Fluo-3 AM and 0.1% Pluronic F-127. For imaging, the cells were washed twice with CIB, and Fluo-3-loaded cells were imaged under the blue light. Unless otherwise specified, drugs were added to the well at 10 s after initial imaging, and responses were monitored at 1-s intervals for an additional 120 s.

2.9. siRNA transfection of LAD2 cells

Specific knockdown was achieved by small interfering RNAs (siRNAs) targeting MRGPRX2 or a negative control siRNA. Smart pools of double-stranded siRNAs targeting MRGPRX2 and nonspecific siRNAs were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences were as follows: Negative Control (NC) forward 5′-UUCUCCGAACGUGUCACGUTT-3′ and reverse 5′-ACGUGACACGUU CGGAGAATT-3′; MRGPRX2-Homo-431 (431) forward 5′-GUACAACAG

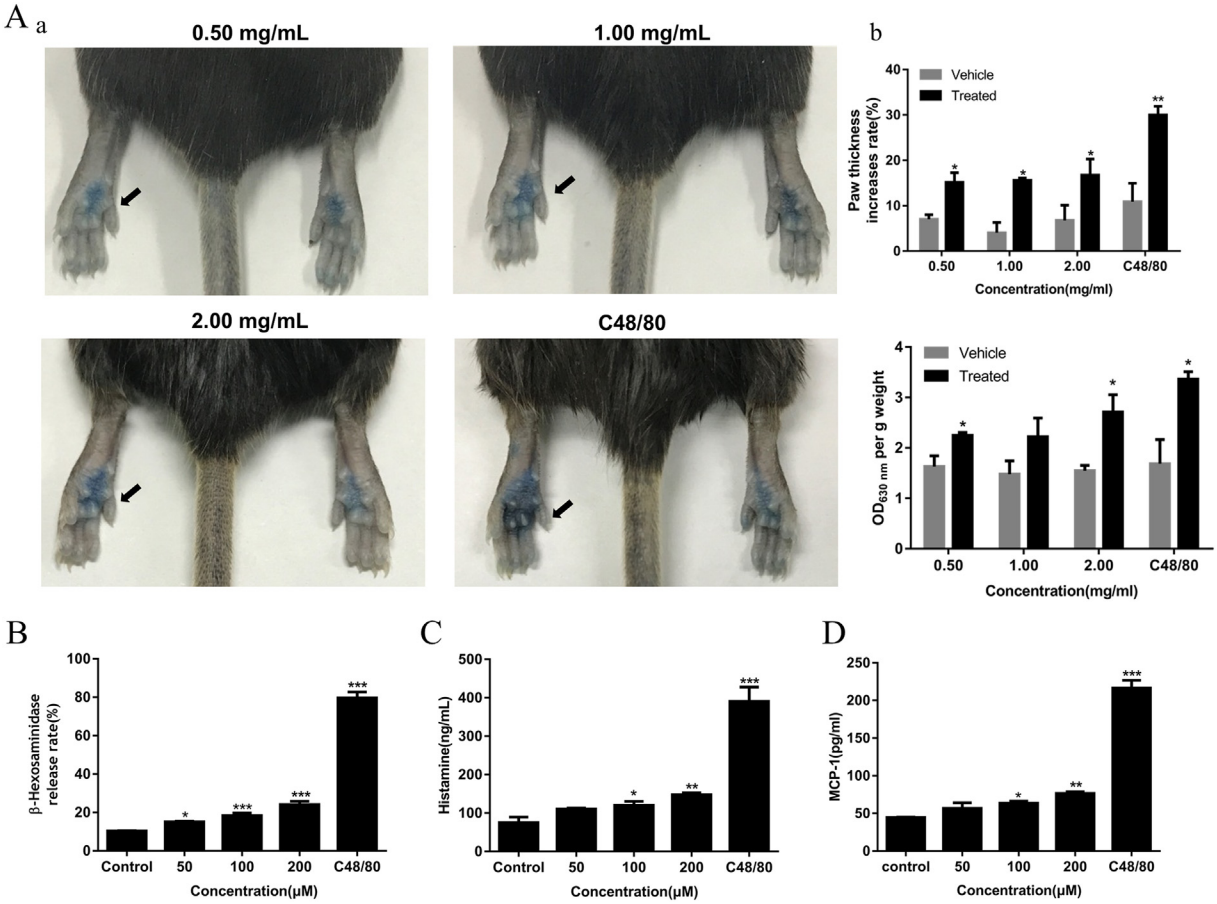

Fig. 1. Isosal C induces pseudo-allergic reactions.

A. Evans blue extravasation into the paws of mice mediated by Isosal C. (n = 6).

a. Representative images of Evans blue-stained extravasation 15 min after the intraplantar injection of 0.5 mg/mL Isosal C, 1.0 mg/mL Isosal C, 2.0 mg/mL Isosal C or 30 μg/mL C48/80 in the left paw or saline in the right paw.

b. Quantification of increased paw thickness and Evans blue leakage into the paw after 15 min.

B. β-Hexasaminidase of LAD2 cells treated with Isosal C for 30 min. (n = 3).

C. Histamine release of LAD2 cells treated with Isosal C for 30 min. (n = 3).

D. MCP-1 release of in LAD2 cells treated with Isosal C for 12 h. (n = 3).

The data are presented as the mean ± S.E.M. Two-tailed unpaired Student's *t*-tests were used to determine significance in panel A, and multiple group comparisons were performed using ANOVA with a Dunnett's post hoc test in panels B–D. Statistical significance was accepted at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

UGAAUGGAAATT-3′ and reverse 5′-UUUCCAUUCACUGUUGUACTT-3′; MRGPRX2-Homo-899 (899) forward 5′-GUGGCUUCUUAUUUAGUG ATT-3′ and reverse 5′-UCACUAAAUAAGAAGCCACTT-3′; and MRGPRX2-Homo-1124 (1124) forward 5′-GGUUCCUAAUAUUAUGGA UTT-3′ and reverse 5′-AUCCAUAAUAUUAGGAACCTT-3′. The siRNAs were delivered at a final concentration of 1 μM by Lipofectamine® 2000 transfection reagent in accordance with the manufacturer's instructions. The cells were incubated for 48 h to allow for MRGPRX2 knockdown.

2.10. β-Hexosaminidase release assay

LAD2 cells were seeded into a 96-well plate at 3×10^4 cells per well and incubated overnight at 37 °C with 5% CO_2. After centrifugation at 200 × *g* for 5 min, the culture medium was removed. Isosal C, at the indicated concentrations diluted in modified Tyrode's solution (120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, 5.5 mM glucose, and 5 mM BSA), was added into the wells (90 μL per well) and incubated for 30 min at 37 °C with 5% CO_2. The 96-well plate was centrifuged at 200 × *g* for 5 min, and the resulting supernatants (50 μL per well) were removed and placed in new wells. To analyze the total β-hexosaminidase content in the cells, cells were lysed with 0.1% Triton X-100 in modified Tyrode's solution. The β-hexosaminidase released into the supernatants and cell lysates was quantified via hydrolysis of *p*-nitrophenyl *N*-acetyl-β-D-glucosamide in 0.1 M sodium carbonate/sodium bicarbonate (pH 11.0) for 90 min at 37 °C, and the samples were measured at 405 nm by a microplate reader. Modified Tyrode's solution was the negative control, and compound 48/80 (30 μg/mL) was used as a positive control.

2.11. Histamine release assay

The histamine release of all samples was measured according to the methods used in our previous studies [18]. In brief, as the β-hexosaminidase release assay, after the LAD2 cells incubated with drugs for 30 min, the supernatants (50 μL per well) were collected and mixed with 100 μL 5 ng/mL d4-HA acetonitrile solution on ice. After centrifuging (12,000 × *g*, 20 min, 4 °C), the supernatants were collected for HPLC-ESI-MS/MS analysis. In the performed HPLC-ESI-MS/MS method of histamine test, isocratic elution on a Venusil HILIC column (150 mm × 2.1 mm I.D., 3 μm, Agela Technologies, Tianjin, China) was employed, the mobile phase consisted of the acetonitrile: water added with 0.1% formic acid and 20 mM ammonium formate (82: 18 v/v) at a

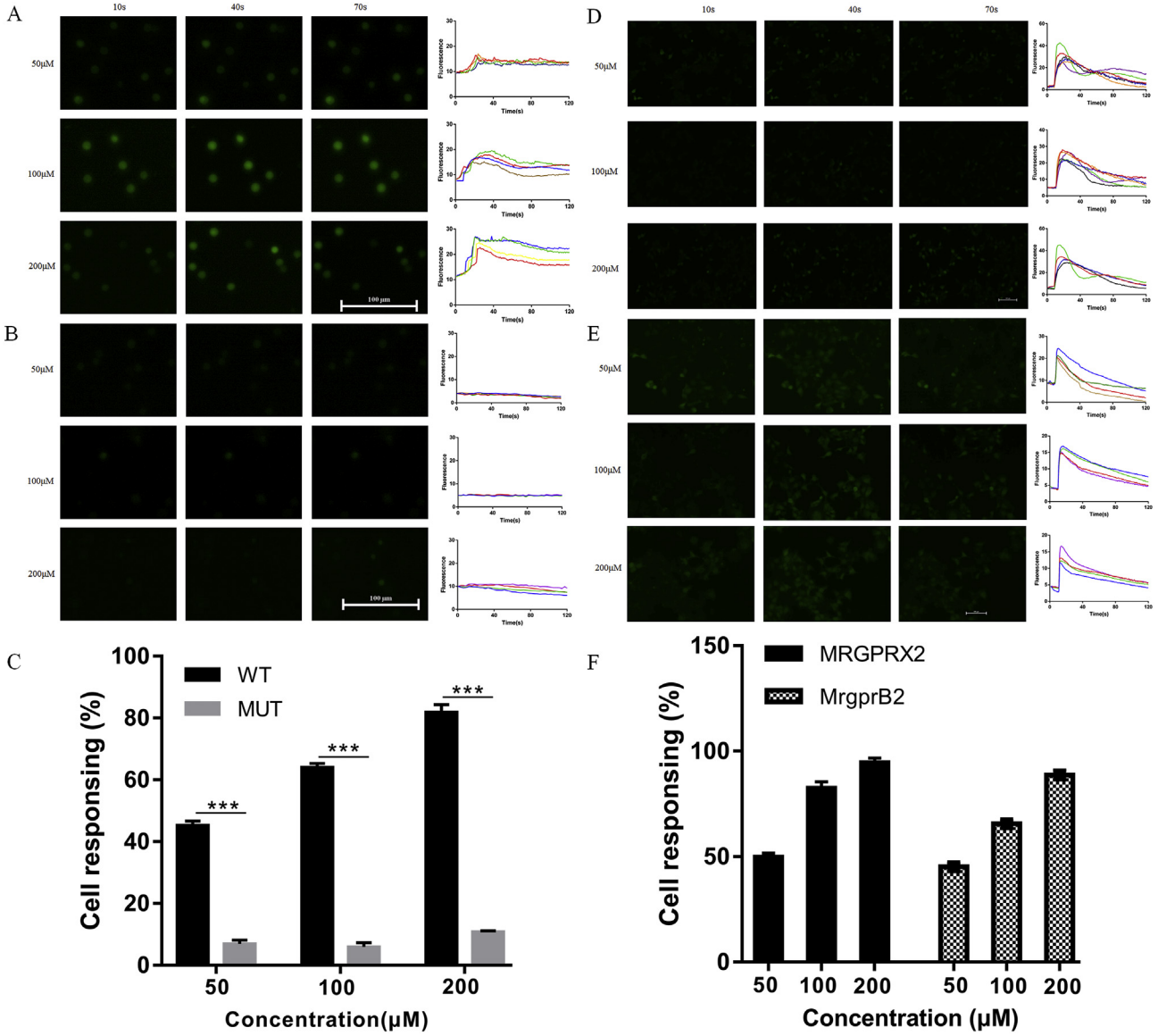

Fig. 2. Calcium imaging of Isosal C in MPMCs, HEK293-MrgprB2 and HEK293-MRGPRX2 cells.

A. Left, representative Fluo-3 fluorescence heat map images of mouse peritoneal MCs showing changes in $[Ca^{2+}]i$ induced by the bath application of 50 μM, 100 μM and 200 μM Isosal C (the images of 10 s was taken before drug stimulation). Right, representative imaging traces and the quantification of responding cells. Each colored line represents an individual cell ($n = 3$ per concentration).

B. Left, representative Fluo-3 fluorescence heat map images of in MUT MPMCs showing changes in $[Ca^{2+}]i$ induced by the bath application of 50 μM, 100 μM and 200 μM Isosal C (the images of 10 s was taken before drug stimulation). Right, representative imaging traces and the quantification of responding cells. Each colored line represents an individual cell (n = 3 per concentration).

C. The cell response rates of WT and MUT MPMCs under Isosal C treatment (n = 3 per concentration).

D. Left, representative Fluo-3 fluorescence heat map images of HEK293-MrgprB2 cells showing changes in $[Ca^{2+}]i$ induced by the bath application of 50 μM, 100 μM and 200 μM Isosal C (the images of 10 s was taken before drug stimulation). Right, representative imaging traces and the quantification of responding cells. Each colored line represents an individual cell (n = 3 per concentration).

E. Left, representative Fluo-3 fluorescence heat map images of in HEK293-MRGPRX2 cells showing changes in $[Ca^{2+}]i$ induced by the bath application of 50 μM, 100 μM and 200 μM Isosal C (the images of 10 s was taken before drug stimulation). Right, representative imaging traces and the quantification of responding cells. Each colored line represents an individual cell (n = 3 per concentration).

F. The cell response rates of HEK293-MrgprB2 and HEK293-MRGPRX2 cells under Isosal C treatment ($n = 3$ per concentration).

The data are presented as the mean ± S.E.M. (n = 3). Two-tailed unpaired Student's *t*-tests were used to determine significance in statistical comparisons, and statistical significance was accepted at $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

flow rate of 0.3 mL min^{-1}.

2.12. Measurement of monocyte chemotactic protein 1

Levels of monocyte chemotactic protein 1 (MCP-1), a chemotactic factor, in cell supernatants were measured by human chemokine array kits according to the manufacturers' instructions after cells were incubated with Isosal C, C48/80 or vehicle for 12 h.

2.13. Molecular docking analysis

To investigate the interactions between MRGPRX2 and Isosal C, a molecular docking assay was performed using Surflex-DockMode of the SYBYL-X 2.0 program package (Tripos, St. Louis, MO, USA). The

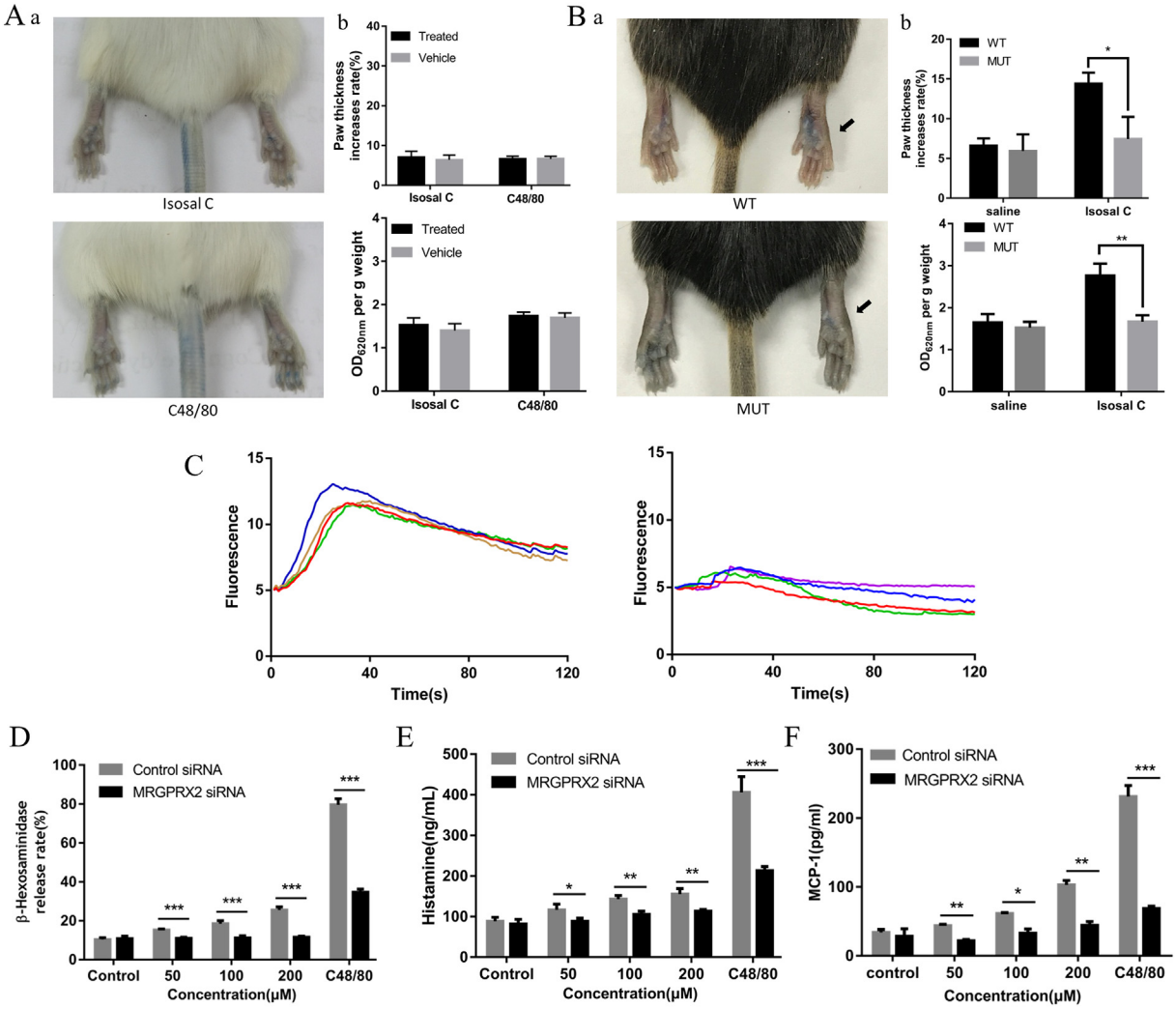

Fig. 3. Isosal C induces pseudo-allergic reactions mediated by MrgprB2 and MRGPRX2.
A. Evans blue extravasation into the paws of C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice mediated by Isosal C. (n = 6).
a. Representative images of Evans blue-stained extravasation 15 min after the intraplantar injection of 2.0 mg/mL Isosal C and 30 μg/mL C48/80.
b. Quantification of increased paw thickness and Evans blue leakage into the paw after 15 min.
B. Evans blue extravasation in the paws of WT and MUT mice. (n = 6).
a. Representative images of Evans blue-stained extravasation 15 min after the intraplantar injection of 0.5 mg/mL Isosal C.
b. Quantification of increased paw thickness and Evans blue leakage into the paw after 15 min.
C. Representative imaging traces of Ca^{2+} concentrations of NC-LAD2 and MRGPRX2 knockdown LAD2 cells treated with 100 μM Isosal C. (n = 3)
D–F. β-Hexasaminidase and histamine release of NC-LAD2 and MRGPRX2 knockdown LAD2 cells treated with Isosal C for 30 min, and MCP-1 release treated with Isosal C for 12 h. (n = 3).
The data are presented as the mean ± S.E.M. Two-tailed unpaired Student's t-tests were used to determine significance in statistical comparisons. Statistical significance was accepted at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

docking model of MRGPRX2 used in this study was based on the predicted MRGPRX2 structure in complex with ZINC-3573 reported by Dr. Bryan Roth [5], and the ZINC-3573 was used to define the binding cavity. The Tripos force field and Pullman charges were employed to add Hydrogen and minimize. Isosal C was depicted by the Sybyl/Sketch module (Tripos Inc.), optimized using Powell's method and the Tripos force field with a 0.05 kcal/(Å mol) convergence criterion, and assigned with the Gasteiger–Hückel method. Other docking parameters were kept at default.

2.14. Frontal analysis

The dissociation equilibrium constant (K_D) is an important affinity parameter for studying drug-receptor interactions, and the frontal analysis was usually utilized to determine the K_D values [19–21]. The frontal analysis is mainly implemented by dissolving the drug into the mobile phase without injection. In our frontal analysis studies, each column was first equilibrated with water (n = 3). The mobile phase was then switched to the aqueous solution, which contained a known concentration of Isosal C. These solutions included up to seven concentrations ranging from 5×10^{-7} to 1×10^{-5} M. Each Isosal C solution was continuously applied to the column until a breakthrough curve with a level plateau was produced. The system was later switched back to water to elute the retained Isosal C from the column under isocratic conditions, and the K_D values can be determined by analyzing the series of breakthrough curves. In the CMC model, the K_D value is investigated by frontal analysis using the following equation:

$$1\text{mLapp} = KD\ \text{mL}\cdot 1\ [\text{A}] + 1\ \text{mL} \quad (1)$$

where mLapp represents the moles of analyte required to reach the

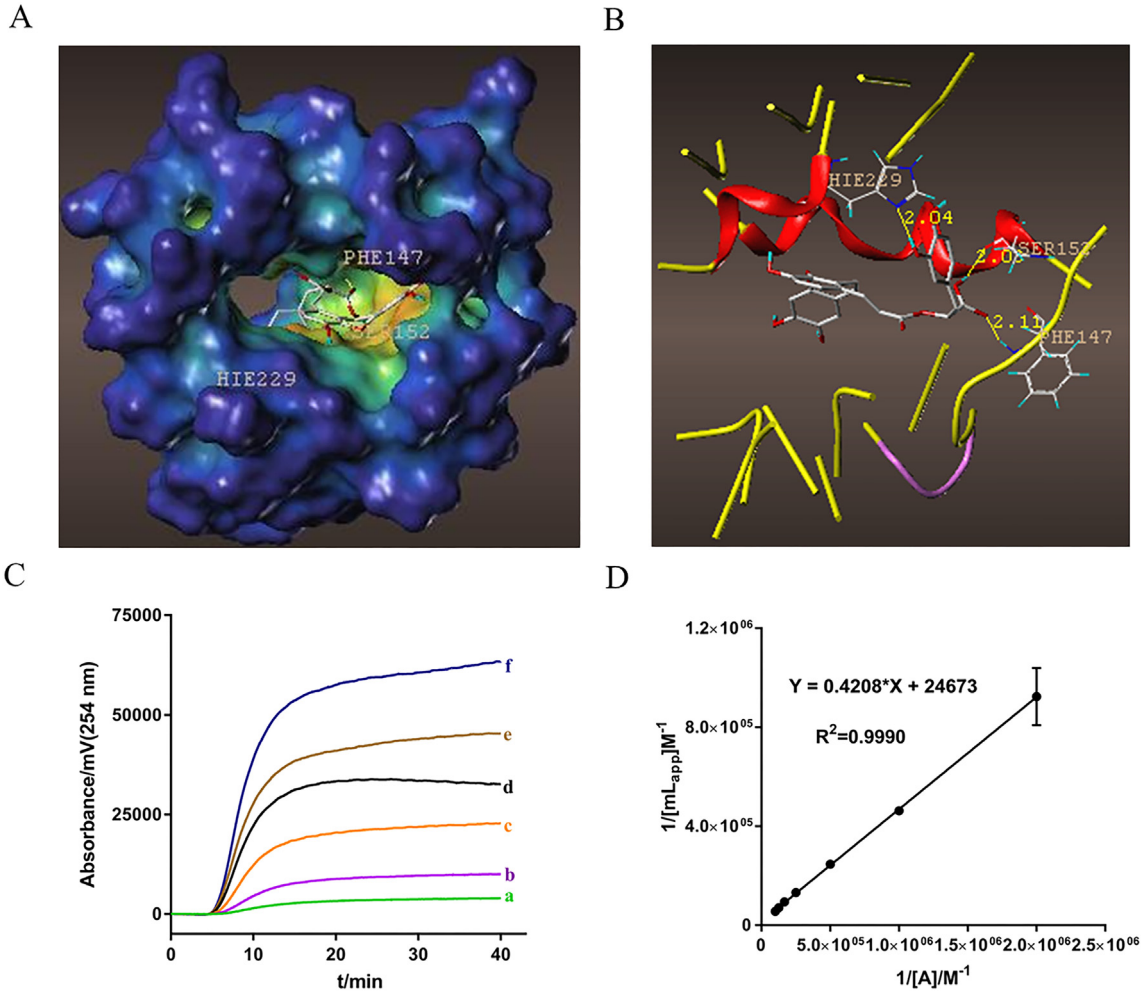

Fig. 4. Molecular docking modeling and the K_D value of Isosal C with MRGPRX2.
A. Sphere space field model of Isosal C and MRGPRX2.
B. Ribbon model of Isosal C and MRGPRX2.
C. The drug concentrations were 2.5×10^{-8}, 5×10^{-8}, 1×10^{-7}, 2×10^{-7}, 4×10^{-7}, 8×10^{-7} and 1×10^{-6} M (from bottom to top), respectively. (n = 3)
D. Regression curves achieved by plotting mLapp versus 1/[A].

mean point of the breakthrough curve at a given molar concentration of the applied analyte and where [A], K_D and mL are the molar concentration of applied analyte, the dissociation equilibrium constants for the analyte at binding sites, and the total moles of binding sites in the column, respectively.

Based on Eq. (1), a plot of 1/mLapp versus 1/[A] should produce a linear relationship for a system, where the value of K_D is found by taking the ratio of the slope over the intercept.

2.15. Western blot analysis

Total protein in untreated and Isosal C-treated LAD2 cells (16 h) was extracted on ice for 30 min using RIPA lysis buffer containing 10% protease inhibitor and a phosphatase inhibitor cocktail (Roche Diagnostics). Insoluble protein lysate was removed by centrifuging the samples at 13,500 × *g* at 4 °C for 10 min. The protein concentration was determined by a BCA protein quantification kit according to the manufacturer's instructions. After the protein in the cell lysates was denatured by boiling the samples with 5 × loading sample buffer (ThermoFisher Scientific, Inc.; MA, USA) for 5 min, equal amounts of protein were separated on a 10% gel using SDS-PAGE (Shaanxi Pioneer Biotech Co., Ltd.). Following electrophoresis, the separated proteins were transferred onto polyvinylidene fluoride membranes (Hangzhou Microna Membrane Technology Co., Ltd.; Hangzhou, China), which were blocked with 5% non-fat milk in Tris-buffered saline (Baihao) containing Tween-20 (TBST; Shaanxi Pioneer Biotech Co., Ltd.) for 2 h at room temperature with continuous agitation. The membranes were then incubated overnight at 4 °C with the following primary antibodies: rabbit anti-PLCγ1 (1:1000, #5690, Cell Signaling Technology (CST)), rabbit anti-phosphorylated-PLCγ1 (P-PLCγ1, Ser1248) (1:1000, #8713, CST), rabbit anti-IP3R (1:1000, #3763, CST), rabbit anti-phosphorylated-IP3R (P-IP3R, Ser1756) (1:1000, #8548, CST), mouse anti-PKC (1:1000, #ab23511, Abcam, this antibody detects PKC alpha, PKC beta and PKC gamma only), rabbit anti-phosphorylated PKC (P-PKC, Ser1756,) (1:500, #9371, CST), rabbit anti-P38 (1:1000, #8690, CST), rabbit anti-phosphorylated-P38 (P—P38, Thr180/Tyr182) (1:1000, #4511, CST), and mouse anti-GAPDH (1:2000, #2118, CST). The membranes were then washed three times with TBST every 10 min followed by incubation with secondary antibodies at a dilution of 1:20,000 in TBST for 1 h at 37 °C. Next, the membranes were washed three times with TBST for 10 min and developed using an enhanced chemiluminescence kit. A Lane 1DTM transilluminator (Beijing Creation Science Co., Ltd., Beijing, China) was used to image the developed blots, and Image-Pro Plus 5.1 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to quantify the protein levels.

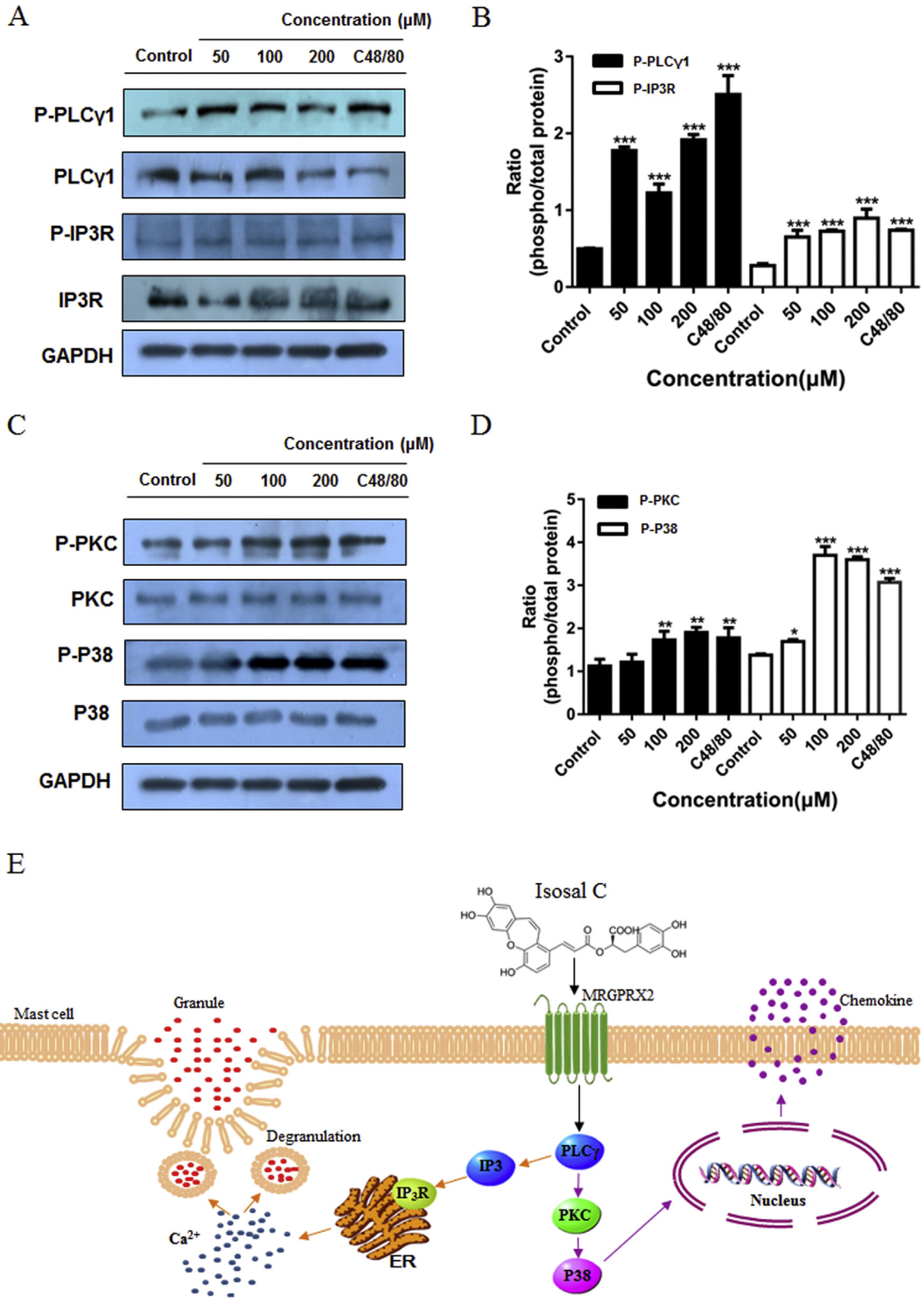

Fig. 5. Effects of Isosal C on the expression of PLC-γ, IP3R, PKC and P38 in LAD2 cells.
A. Western blot analysis of the expression levels of PLCγ1, IP3R, phosphorylated PLCγ1 and phosphorylated IP3R in LAD2 cells treated with Isosal C.
B. Quantification of PLCγ1, IP3R, phosphorylated PLCγ1 and phosphorylated IP3R protein expression by densitometric analysis.
C. Western blot analysis of PKC, P38, phosphorylated PKC and phosphorylated P38 expression levels in LAD2 cells treated with Isosal C.
D. Quantification of PKC, P38, phosphorylated PKC and phosphorylated P38 protein expression by densitometric analysis.
E. The molecular signaling pathways mediated isosalvianolic acid C-induced pseudo-allergic reactions.
The data are presented as the mean ± S.E.M. (n = 3). Multiple group comparisons were performed using ANOVA with a Dunnett's post hoc test, and statistical significance was accepted at $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

2.16. Statistical analysis

The group data were expressed as the mean ± S.E.M and assessed for normality with Shapiro-Wilk test. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) with a Dunnett's post hoc test, post hoc tests were only performed if F was significant. Two-tailed unpaired Student's t-test was used to conduct all analyses between both groups. In addition, the analyses were carried out using SPSS software and GraphPad Prism version 7.0. Differences were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Pseudo-allergic reactions in Isosal C-induced occur

To explore the pseudo-allergic reactions of Isosal C in vivo, we first used an in vivo model of systemic anaphylaxis (Fig. s1). The results show that the systemic anaphylaxis of mice will occur under 0.52 mg/kg Isosal C treatment, and the leakage of Evans blue into the ear was increased in a dose-dependent manner under Isosal C treatment. Then a Hindpaw swelling and extravasation assay was also employed. Subcutaneous injection of Isosal C (left foot) triggered hindpaw inflammation (extravasation and swelling) in mice in a dose-dependent manner, while saline (vehicle) did not trigger inflammation, and the C48/80 was used as the positive control (Fig. 1A). Furthermore, mast cell degranulation assays were employed to evaluate the pseudo-allergic reactions of Isosal C in vitro. As expected, in LAD2 MCs, 0, 50 μM, 100 μM, and 200 μM Isosal C and 30 μg/mL C48/80 promoted the secretion of β-hexosaminidase (Fig. 1B), histamine (Fig. 1C), and MCP-1 (respectively; Fig. 1D) in a dose-dependent manner.

3.2. Isosal C increases calcium mobilization in MPMCs via MrgprB2

MPMCs have been reported to express MrgprB2 in a highly specific manner [5,7,22]. Calcium imaging of MPMCs showed an increase in intracellular Ca^{2+} release in a dose-dependent manner when treated with 50 μM, 100 μM and 200 μM Isosal C with cell response rates of 45.29% ± 0.0130, 64.02% ± 0.0131 and 81.89% ± 0.0237, respectively (Fig. 2A). In contrast, MUT MPMCs treated with 50 μM, 100 μM and 200 μM Isosal C showed almost no increase in intracellular Ca^{2+} release with cell response rates of 6.99% ± 0.117, 5.92% ± 0.0137 and 10.91 ± 0.0018, respectively (Fig. 2B), the cell response rates is significantly reduced than the MPMCs of MrgprB2 (WT) mice (Fig. 2C). These results indicated that Isosal C increases calcium mobilization in MPMCs via MrgprB2.

3.3. Isosal C induces calcium mobilization in HEK293-MRGPRX2 cells and HEK293-MrgprB2 cells

HEK293-MRGPRX2 (highly express MRGPRX2) and HEK293-MrgprB2 (highly express MrgprB2) cells express targets of many small molecular drugs associated use to treat systemic pseudo-allergic reactions. HEK293-MRGPRX2 cells and HEK293-MrgprB2 cells were treated with 50 μM, 100 μM, and 200 μM Isosal C (Fig. 2D, E), resulting in a significant increase in calcium mobilization (cell response rates of 49.79% ± 0.0189, 82.42% ± 0.0302 and 94.50% ± 0.0216, respectively, for HEK293-MRGPRX2 cells; 45.24% ± 0.0148, 65.51% ± 0.0139 and 88.73% ± 0.0221, respectively, for HEK293-MrgprB2 cells) (Fig. 2F). These results demonstrated that Isosal C induces calcium mobilization in HEK293-MRGPRX2 cells and HEK293-MrgprB2 cells via MRGPRX2 and MrgprB2.

3.4. Isosal C induced pseudo-allergic reactions in mice via Mrgprb2

To verify that the Isosal C-induced hindpaw inflammation was dependent on MCs, a C57BL/6-KitW^{-sh}/W^{-sh} mice model was employed. Isosal C-induced hindpaw inflammation was almost completely absent in C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice (Fig. 3A), which indicated that Isosal C-induced pseudo-allergic reactions were associated with MCs. Further, MrgprB2$^{-/-}$ mice were used to investigate whether Isosal C-induced hindpaw inflammation was MrgprB2-dependent. Compared to MrgprB2 (WT) mice, MrgprB2$^{-/-}$ mice showed almost no hindpaw inflammation (Fig. 3B), which indicated that the Isosal C-induced activation of MC mediator release and vasodilation in vivo was associated with MrgprB2 on MCs.

3.5. MRGPRX2 mediates Isosal C-induced MC degranulation and release of chemokines

To determine whether MRGPRX2 mediates the MC degranulation induced by Isosal C, LAD2 and MRGPRX2 knockdown LAD2 cells were used to investigate changes in intracellular Ca^{2+} and the secretion of β-hexosaminidase, histamine and MCP-1 (degranulation marker, mediator of increased vascular permeability and chemokine, respectively). The knockdown efficiency of MRGPRX2 knockdown LAD2 cells was evaluated by western blot and RT-PC, and the results (Fig. s2) verified the knockdown MRGPRX2 was completed. The calcium imaging assay showed that Isosal C led to significant decrease intracellular Ca^{2+} levels in MRGPRX2 knockdown LAD2 cells than the NC-transfected LAD2 cells (Fig. 3C), shows that MRGPRX2 mediates the calcium mobilization in LAD2 cells induced by Isosal C. when it comes to degranulation and release of chemokines induced by Isosal C, NC-transfected LAD2 cells showed that 0, 50 μM, 100 μM, and 200 μM Isosal C and 30 μg/mL C48/80 promoted the secretion of β-hexosaminidase, histamine and MCP-1 in a dose-dependent manner. MRGPRX2 knockdown LAD2 cells showed that 0, 50 μM, 100 μM, and 200 μM Isosal C and 30 μg/mL C48/80 promoted the secretion of β-hexosaminidase, histamine and MCP-1, the release of β-hexosaminidase, histamine and MCP-1 from MRGPRX2 knockdown LAD2 cells decreased significantly than LAD2 cells and NC-transfected LAD2 cells. These results revealed that Isosal C induces calcium mobilization and degranulation in LAD2 cells via MRGPRX2.

3.6. Molecular docking and frontal analysis reveals the interaction between Isosal C and MRGPRX2

To investigate the interactions between Isosal C and MRGPRX2, molecular docking was performed using Surflex-Dock mode of the SYBYL-X 2.0 program package. The docking results showed that Isosal C matched the active pocket of MRGPRX2 well in the sphere space field model (Fig. 4A). Detailed analysis of the interaction between Isosal C and MRGPRX2 is shown in Fig. 4B. Based on the docking results, Isosal C formed three hydrogen bonds with MRGPRX2. The carbonyl oxygen in the carboxyl group of Isosal C formed one hydrogen bond with the amino hydrogen of PHE147 with a length of 2.11 Å. The hydroxyl hydrogen in the carboxyl group of Isosal C and the carbonyl oxygen of SER152 formed one hydrogen bond with a length of 2.03 Å. The hydroxyl hydrogen formed one hydrogen bond with the imidazole nitrogen of HIE229 with a length of 2.04 Å. These results suggested that Isosal C binds well to MRGPRX2.

The K_D of drug-membrane receptor affinity is the basic parameter that reflects the strength of an interaction, and cell membrane chromatography is an effective technique to study the characteristics of drug-membrane receptor affinity. In this study, the K_D value of Isosal C-MRGPRX2 was obtained by frontal analysis (Fig. 4C, D) and found to be $12.77 \pm 0.54 \times 10^{-5}$ ($R^2 = 0.9992$), indicating the strength of Isosal C-MRGPRX2 affinity.

3.7. Isosal C activates PLC-γ, IP3R, PKC and P38 to trigger pseudo-allergic reactions

Activation of PLC-γ induces the IP3 produce, binding to IP3R causes

the instantaneous release of Ca^{2+} from endoplasmic reticulum (ER) stores. Activating PLC-γ also activates PKC and P38, which is closely related to chemokine production and release in MCs [7]. Therefore, we investigated whether Isosal C activates PLCγ, IP3R, PKC and P38 in LAD2 cells by performing western blotting. The results showed that P-PLC-γ, P-IP3R, P-PKC and P−P38 levels were increased in a dose-dependent manner when LAD2 MCs were treated with 50 μM, 100 μM and 200 μM Isosal C for 16 h (Fig. 5A–D). These results indicated that Isosal C triggers pseudo-allergic reactions by activating PLC-γ, IP3R, PKC and P38 (Fig. 5E).

4. Discussion

It was found that Isosal C could cause mast cell degranulation in previous work [17]. In this study, we firstly identified and showed that Isosal C induces pseudo-allergic reactions via the MC-specific receptor MRGPRX2 and the PLC molecular signaling pathways.

In vivo study, we revealed that Isosal C triggers systemic anaphylaxis of mice. Therefore, we investigated local anaphylaxis associated with Isosal C and found that Isosal C induces local anaphylaxis in mice (Fig. 1A). In vitro studies, we found that Isosal C caused LAD2 cells degranulation (Fig. 1B, C). In addition, Isosal C stimulated the LAD2 cells to synthesize and excrete chemotactic factor MCP-1 (Fig. 1D). Based on the in vitro and in vivo results, we confirmed that Isosal C induces anaphylactoid reactions.

To confirm the relationship between MRGPRX2 and Isosal C in pseudo-allergic reactions, we analyzed the effects of Isosal C on changes in Ca^{2+} mobilization in MPMCs, and found that Isosal C induced changes in intracellular Ca^{2+} concentrations in MPMCs trough MrgprB2 (Fig. 2A–C). We also analyzed the effects of Isosal C on changes in calcium mobilization in HEK-MrgprB2 and HEK-MRGPRX2 cells, and confirmed that Isosal C induce anaphylactoid reactions via MRGPRX2 and MrgprB2. To certify Isosal C induces anaphylactoid reaction through MRGPRX2 in vivo, we used C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice and MUT mice to compare the local pseudo-allergic reactions induced by Isosal C, and confirmed that Isosal C induces pseudo-allergic MC-dependent reactions via MrgprB2 in vivo (Fig. 3A, B). In addition, we used MRGPRX2 knockdown LAD2 MCs as a model system to further confirm that Isosal C induces MC degranulation as well as chemotactic factor synthesis and excretion via MRGPRX2 in vitro (Fig. 3C–F). All the results confirmed that Isosal C induced pseudo-allergic reactions via MRGPRX2. To further explore the interaction between Isosal C and MRGPRX2, molecular docking and K_D studies of Isosal C-MRGPRX2 were performed (Fig. 4). Isosal C bound MRGPRX2 well, indicating that Isosal C act on MRGPRX2.

In 2015, Grimbaldeston found that there are differences between the MRGPRX2- and IgE-FcεRI-induced MC degranulation signaling pathways [23]. In this study, we found that Isosal C regulates intracellular calcium fluctuations via MRGPRX2-PLC signaling and induces MC degranulation. This study indicated that the interaction between MRGPRX2 and Isosal C causes PLCγ1 and IP3R phosphorylation in MCs (Fig. 5A, B, E). Prolonged stimulation with Isosal C also phosphorylated PKC and P38, thereby promoting the synthesis of pro-inflammatory cytokines (Fig. 5C, D, E).

In this study, the pseudo-allergic reactions of Isosal C were confirmed. This study is also the first to confirm that MRGPRX2 and its murine homologue, MRGPRB2, are the only receptors that mediate Isosal C-induced pseudo-allergic reactions. In addition, the mechanism underlying Isosal C-induced pseudo-allergic reactions was also firstly investigated using western blotting assays. Base on the research in our study, drugs containing Isosal C should be followed in the process of production and clinical use to ensure the safe dosage.

Conflict of interest statement

The authors declare no conflicts of interest related to this work.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant Number: 81503032, 81230079, and 81227802), National China Postdoctoral Science Foundation Funded Project (Grant Number: 2015M570844, and 2016T90930), Natural Science Foundation of Shaanxi Province (Grant Number: 2017JM8050), Shaanxi Province Postdoctoral Science Foundation Funded Project and the Fundamental Research Funds for the Central Universities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.03.013.

References

[1] J.K. Lee, P. Vadas, Anaphylaxis: mechanisms and management, Clin. Exp. Allergy 41 (2011) 923–938.
[2] H. Subramanian, K. Gupta, H. Ali, Roles of Mas-related G protein-coupled receptor X2 on mast cell-mediated host defense, pseudoallergic drug reactions, and chronic inflammatory diseases, J. Allergy Clin. Immunol. 138 (2016) 700–710.
[3] F.D. Finkelman, M.V. Khodoun, R. Strait, Human IgE-independent systemic anaphylaxis, J. Allergy Clin. Immunol. 137 (2016) 1674–1680.
[4] B.D. McNeil, P. Pundir, S. Meeker, L. Han, B.J. Undem, M. Kulka, X.Z. Dong, Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions, Nature 519 (2015) 237–241.
[5] K. Lansu, J. Karpiak, J. Liu, X.P. Huang, J.D. McCorvy, W.K. Kroeze, T. Che, H. Nagase, F.I. Carroll, J. Jin, B.K. Shoichet, B.L. Roth, In silico design of novel probes for the atypical opioid receptor MRGPRX2, Nat. Chem. Biol. 13 (2017) 529–536.
[6] T. Zhang, D.L. Che, R. Liu, S.L. Han, N. Wang, Y.Z. Zhan, P. Pundir, J. Cao, Y.N. Lv, L. Yang, J. Wang, M.W. Ding, X.Z. Dong, L.C. He, Typical antimicrobials induce mast cell degranulation and anaphylactoid reactions via MRGPRX2 and its murine homologue MRGPRB2, Eur. J. Immunol. 47 (2017) 1949–1958.
[7] R. Liu, D.L. Che, T. Zhang, P. Pundir, J. Cao, Y.N. Lv, J. Wang, P.Y. Ma, J. Fu, N.N. Wang, X.Y. Wang, T. Zhang, X.Z. Dong, L.C. He, MRGPRX2 is essential for sinomenine hydrochloride induced anaphylactoid reactions, Biochem. Pharmacol. 146 (2017) 214–223.
[8] T.X. Qian, L.N. Li, Isosalvianolic acid C, a depside possessing a dibenzooxepin skeleton, Phytochemistry 31 (1992) 1068–1070.
[9] D.A. Guo, W.Y. Wu, M. Ye, X. Liu, G.A. Cordell, A holistic approach to the quality control of traditional Chinese medicines, Science 347 (2015) S29–S31.
[10] Y.G. Li, L. Song, M. Liu, Z.B. Hu, Z.T. Wang, Advancement in analysis of *Salviae miltiorrhizae Radix et Rhizoma* (Danshen), J. Chromatogr. A 1216 (2009) 1941–1953.
[11] S.P.M. Ventura, F.A.E. Silva, M.V. Quental, D. Mondal, M.G. Freire, J.A.P. Coutinho, Ionic-liquid-mediated extraction and separation processes for bioactive compounds: past, present, and future trends, Chem. Rev. 117 (2017) 6984–7052.
[12] Y.L. Lin, Y.Y. Chang, Y.H. Kuo, M.S. Shiao, Anti-lipid-peroxidative principles from Tournefortia sarmentosa, J. Nat. Prod. 65 (2002) 745–747.
[13] J.K. Dhanjal, S. Sharma, A. Grover, A. Das, Use of ligand-based pharmacophore modeling and docking approach to find novel acetylcholinesterase inhibitors for treating Alzheimer's, Biomed. Pharmacother. 71 (2015) 146–152.
[14] L. Wang, Q. Yuan, G. Marshall, X. Cui, L. Cheng, Y.Y. Li, H.C. Shang, B.L. Zhang, Y.P. Li, Adverse drug reactions and adverse events of 33 varieties of traditional Chinese medicine injections on National Essential medicines List (2004 edition) of China: an overview on published literatures, J. Evid. Based Med. 13 (2010) 95–104.
[15] S.L. Han, T. Zhang, J. Huang, R.H. Cui, L.C. He, New method of screening allergenic components from Shuanghuanglian injection: with RBL-2H3/CMC model online HPLC/MS system, J. Pharm. Biomed. Anal. 88 (2014) 602–608.
[16] Y.B. Xu, D.Q. Dou, X.K. Ran, C.Y. Liu, J. Chen, Integrative analysis of proteomics and metabolomics of anaphylactoid reaction induced by Xuesaitong injection, J. Chromatogr. A 1416 (2015) 103–111.
[17] Y.Y. Lin, C. Wang, Y.J. Hou, W. Sun, D.L. Che, L. Yang, T. Zhang, M. Sun, H.Z. He, L.C. He, Studies for simultaneous identification of three pseudo-allergic components in Danshen injection by using high expression Mas-related G protein coupled receptor X2 cell membrane chromatography coupled online with HPLC-ESI-MS/MS system, J. Sep. Sci. 41 (2018) 2488–2497.
[18] S.L. Han, Y.N. Lv, L.Y. Kong, D.L. Che, R. Liu, J. Fu, J. Cao, J. Wang, C. Wang, H.Z. He, T. Zhang, X.Z. Dong, L.C. He, Use of the relative release index for histamine in LAD2 cells to evaluate the potential anaphylactoid effects of drugs, Sci. Rep. 7 (2017).
[19] W.N. Ma, L. Yang, Y.N. Lv, J. Fu, Y.M. Zhang, L.C. He, Determine equilibrium dissociation. Constant of drug-membrane receptor affinity using the cell membrane chromatography relative standard method, J. Chromatogr. A 1503 (2017) 12–20.
[20] M. Sanghvi, R. Moaddel, I.W. Wainer, The development and characterization of protein-based stationary phases for studying drug–protein and protein–protein interactions, J. Chromatogr. A 1218 (2017) 8791–8798.
[21] J.J. Slon-Usakiewicz, J.R. Dai, W. Ng, J.E. Foster, E. Deretey, L. Toledo-Sherman,

P.R. Redden, A. Pasternak, N. Reid, Global kinase screening. Applications of frontal affinity chromatography coupled to mass spectrometry in drug discovery, Anal. Chem. 77 (2005) 1268–1274.

[22] A. Solis-Lopez, U. Kriebs, A. Marx, S. Mannebach, W.B. Liedtke, M.J. Caterina, M. Freichel, V.V. Tsvilovskyy, Analysis of TRPV channel activation by stimulation of FCεRI and MRGPR receptors in mouse peritoneal mast cells, PLoS One 12 (2017) e0171366.

[23] M.A. Grimbaldeston, Mast cell-MrgprB2: sensing secretagogues or a means to overreact? Immunol. Cell Biol. 93 (2015) 221–223.