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Biochemical Pharmacology

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

MRGPRX2 is essential for sinomenine hydrochloride induced anaphylactoid reactions

Rui Liu^{a,b}, Delu Che^a, Tingting Zhao^a, Priyanka Pundir^b, Jiao Cao^a, Yanni Lv^a, Jue Wang^a, Pengyu Ma^a, Jia Fu^a, Nana Wang^a, Xiaoyang Wang^a, Tao Zhang^a, Xinzhong Dong^{b,*}, Langchong He^{a,*}^a School of Pharmacy, Xi'an Jiaotong University, Xi'an 710061, China^b The Solomon H. Snyder Department of Neuroscience, Johns Hopkins University, School of Medicine, Baltimore, MD 21205, USA

ARTICLE INFO

Article history:

Received 3 August 2017

Accepted 29 September 2017

Available online xxxxx

Keywords:

Sinomenine hydrochloride (SH)

Mast cell

MRGPRX2

Degranulation

Anaphylactoid reactions

ABSTRACT

Mast cells are unique immunocytes that function as sentinel cells in host defense reactions such as immediate hypersensitivity responses and anaphylactic responses. The mast cell specific receptor MRGPRX2 (Mas-related G protein-coupled receptor X2) triggers mast cell degranulation—a key process in anaphylactic reactions. We sought to better understand anaphylactic reaction induced by sinomenine hydrochloride (SH). MRGPRX2-related pseudo-allergic reactions induced by SH were investigated using the hindpaw swelling and extravasation assay *in vivo* and mast cell degranulation assays *in vitro*. MrgprB2 knockout mice exhibit a reduced SH-induced inflammation effect. Furthermore, MRGPRX2 (the orthologous gene of MrgprB2) related human mast cells are activated by SH in a dose-dependent manner; however, MRGPRX2 knockdown mast cells showed reduced degranulation. The results showed a kind of mechanism that SH-induced anaphylactoid reactions were mediated by MRGPRX2 via activating PLC molecular signaling pathways to provoke mast cells Ca^{2+} mobilization and degranulation.

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1. Introduction

Sinomenine is a natural alkaloid extracted from the root of the traditional Chinese medicinal plant *Caulis sinomenii* and belongs to the family of morphinan alkaloids [1]. It is reported to be a therapeutic administered for rheumatoid arthritis [2], and also possesses immunomodulatory [3], anti-inflammatory [4], anti-hypertensive [5], anti-arrhythmic [6], and other biological properties. In addition, sinomenine mediates certain pharmacological effects such as improving conditions of chronic nephritis [7,8] as well as anti-oxidant [9], analgesic [10], anti-tumor [11,12], and neuroprotective effects [13,14]. Injections of the Zheng Qing Feng Tong Ning preparation and other drugs that utilize sinomenine as the primary active constituent are clinically used to treat rheumatoid arthritis [2,15]. However, with increasing clinical doses, the incidence of reported adverse reactions has increased annually. Clinically, these adverse reactions manifest as skin pruritus, redness and swelling of the cheeks, rash, and other anaphylactoid reactions, which could lead to anaphylactic shock in severe cases

[16]. Therefore, clinical anaphylactoid reactions induced by SH are currently receiving considerable research attention.

Anaphylactoid reactions are a non-immune-mediated allergic reaction with an apparent dose-dependent effect [17,18]. An earlier report by Yamasaki noted that SH can induce histamine release from mast cells in mammalian tissues [19]. Huang et al. reported that in rat RBL-2H3 cells, SH could promote the release of anaphylactic mediator independent of fluctuations in calcium levels [20]. In 2016, Wang et al. demonstrated that SH could regulate calcium levels in RBL-2H3 cell resulting in the release of anaphylactic mediators [21]. To date, the effects of SH on human mast cells have not been studied. In 2015, McNeil et al. were the first to demonstrate that secretagogues and small molecule drugs could activate human LAD2 mast cells by binding to MRGPRX2. This activation increases the intracellular calcium concentration and degranulation, mediates anaphylactoid reactions. Their group also confirmed that MRGPRX2 and mouse MrgprB2 are homologous genes [22]. Molecular docking is considered for predicting novel molecules that bind to a protein, which has been getting more and more accurate [23]. In 2017, Katherine reported that SH can activate LAD2 cells, however, they didn't explain more [24]. Hence, the specific mechanisms by which SH activates mast cells and triggers anaphylactoid reactions is still unclear.

* Corresponding authors at: The Solomon H. Snyder Department of Neuroscience, Johns Hopkins University, School of Medicine, Baltimore, MD 21205, USA (X. Dong). School of Pharmacy, Xi'an Jiaotong University, Yanta West Road, Xi'an 710061, China (L. He).

E-mail addresses: xdong2@jhmi.edu (X. Dong), helc@mail.xjtu.edu.cn (L. He).

In this study, we used the MrgprB2 gene wild-type and knock-out mice, and mast cell-deficient *W-sash c-kit* mutant *Kit^{W-sh/W-sh}* mice to investigate the anaphylactoid effects of SH *in vivo* and observed that SH induces anaphylactoid reactions by activating the MrgprB2 gene on mast cells. We also used human mast cells LAD2 and HEK293 cells that express elevated levels of either MrgprB2 or MRGPRX2 to elucidate the functional target of SH and the mechanism by which calcium mobilization, degranulation, and other steps involved in triggering anaphylactoid reactions *in vitro*. We demonstrated the mechanism by which SH induced anaphylactoid reactions and mast cell activation via MRGPRX2, which triggered changes in calcium influx related protein signaling pathways.

2. Materials and methods

2.1. Drugs and reagents

Compound 48/80 (C48/80, a classical mast cell activator and canonical basic secretagogue), Evans blue and p-nitrophenyl N-acetyl- β -D-glucosamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). SH was obtained from Chengdu Pufei De Biotech Co., LTD (Chengdu, Sichuan, China) and purified to $\geq 98\%$. Saline was purchased from Shandong Qilu Pharmaceutical Co., Ltd (Jinan, Shandong, China). An R&D Proteome Profiler Human XL Cytokine Array Kit and a Human CCL3/MIP-1 β Quantikine ELISA Kit were purchased from Bio-Techne (Minneapolis, MN, USA). A Human IL-8 ELISA Kit, Human MCP-1 ELISA Kit and Human TNF- α ELISA Kit were purchased from ExCell Biology, Inc. (Shanghai, China). Fura-2 AM and Fluo-4 AM were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Pluronic F-127 was procured from Biotium (Fremont, CA, USA).

2.2. Mouse models

C57BL/6 (wild-type [WT]) mice were purchased from the Experimental Animal Center at Xi'an Jiaotong University (Xi'an, China). Young adult mice aged 6–8 weeks were used in all the experiments in this study. MrgprB2-knockout (KO) mice on a C57BL/6 background were kindly provided by Professor Xinzhong Dong from Johns Hopkins University (MD, USA), and mast cell-deficient *W-sash c-kit* mutant *Kit^{W-sh/W-sh}* mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). Animals were housed at the Experimental Animal Center of Xi'an Jiaotong University and provided food and water ad libitum. All experiments requiring identical treatment administration in the animals were conducted by investigators blinded to the conditions.

2.3. Ethics statement

The study was conducted in strict accordance with 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 approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011-0045).

2.4. Cell lines

Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe from NIH (MD, USA). Cells were maintained in StemPro-34 medium supplemented with StemPro nutrient supplement, 100 U of penicillin-streptomycin, 2 mM L-glutamine and 100 ng/ml human stem cell

factor in a 37 °C incubator containing 5% CO₂. Culture medium was replaced every 7 days, and the cells were maintained at a density of 2×10^6 cells/ml. HEK293-MRGPRX2 and HEK293-MrgprB2 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).

2.5. Peritoneal mast cell purification assay

Young adult mice (8–10 weeks of age) were euthanized via CO₂ inhalation. A total of 12 ml of ice-cold mast cell 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 centrifuged at 200g and 4 °C for 5 min. The cells from each mouse were resuspended in 2 ml MCDM, layered them over 4 ml of an isotonic 70% Percoll suspension (2.8 ml of Percoll, 320 μ l of $10 \times$ HBSS, 40 μ l of 1 M HEPES, 830 μ l of MCDM), and centrifuged at 500g and 4 °C for 20 min. The purity of the pelleted mast cells was $>95\%$ as assayed by morphology and toluidine blue staining. Mast cells were resuspended at concentrations of 5×10^5 to 1×10^6 cells/ml in DMEM supplemented with 10% FBS, 100 U of penicillin-streptomycin and 25 ng/ml recombinant mouse stem cell factor (mSCF).

2.6. Hindpaw swelling and extravasation assay

Young adult mice (aged 6–8 weeks old) were anesthetized with an intraperitoneal injection of 70 mg/kg pentobarbital sodium. Fifteen minutes after anesthesia induction, each mouse was intravenously (i.v.) injected with 50 μ l of 12.5% Evans blue in saline, and a Vernier caliper was used to measure the thickness of the paws before any test substances were injection. Five minutes later, 5 μ l of 2.5 mg/ml, 5 mg/ml, or 10 mg/ml SH or 10 μ g/ml C48/80 was injected into one paw, and saline was injected into the other paw as a negative control. Fifteen minutes later, the paw thicknesses were measured again and recorded. Mice were then euthanized by decapitation, and the paw tissues were collected, dried at 50 °C, and weighed separately. Evans blue dye was extracted by adding 500 μ l of a mixture of acetone-saline (7:3) to each tissue sample and incubating at 37 °C for 12 h. Tissues were then minced, disrupted for 10 min in an ultrasonic machine, and centrifuged at 3000 rpm for 20 min. The supernatant was aliquoted equally into 96-well plates (200 μ l/well), and the OD value at 620 nm was measured using a Microplate Reader.

2.7. The β -hexosaminidase release assay

LAD2 cells were seeded into a 96 well plate at 2×10^4 cells per well and incubated overnight. Then, the culture medium was removed, either SH or C48/80 at the indicated concentrations diluted in modified Tyrode's solution (120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM HEPES, 5.5 mM glucose, 5 mM BSA) was added to the wells and incubated for 30 min. The 96-well plate was centrifuged at 200g for 5 min. To analyze total β -hexosaminidase content in the cells, the cells were lysed with 0.1% Triton X-100 in modified Tyrode's solution, and the β -hexosaminidase that was released into the supernatants and cell lysates was quantified via 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 halted by adding stop buffer (0.1 M sodium carbonate/sodium bicarbonate, pH 11.0), and the samples were measured at 405 nm using a Microplate Reader. C48/80 was used as a positive control.

2.8. Histamine release assay

Histamine (HA) was purchased from Sigma, histamine-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 (Waltham, MA, USA). LC-MS grade formic acid was obtained from Sigma. An LCMS-8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used in the applied LC-ESI-MS/MS method. Histamine was evaluated on this system with an HILIC column (Venusil HILIC, 2.1 mm × 150 mm, 3 μm, Agela Technologies, Tianjin, China), and an isocratic elution buffer comprising a solution of acetonitrile–water containing 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) was used to elute the histamine at a flow rate of 0.3 ml/min.

2.9. Intracellular Ca^{2+} mobilization assay

All the drugs were diluted to the required concentration by 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, brought to pH 7.4 using NaOH). The incubation buffer comprised 4 μM of either Fluo-4 AM or Fura-2 AM and 0.1% Pluronic F-127. For imaging, the cells were washed twice in CIB, and Fluo-4 AM or Fura-2 AM loaded cells were imaged at excitation wavelengths of 488 nm or 340/380 nm, respectively. 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.

2.10. siRNA transfection of LAD2 cells

Specific knockdown was achieved using either small interfering RNAs (siRNAs) targeting MRGPRX2 or a negative control siRNA. A smart pool of double-stranded siRNAs targeting MRGPRX2 as well as non-specific siRNAs was obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences were as follows: Negative Control siRNA, forward, 5'-UUCUCCGAACGUGUCAC GUTT-3', and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'; MRGPRX2 knockdown siRNA, forward, 5'-GUACAACAGUGAUG GAAATT-3', and reverse, 5'-UUUCCAUCACUGUUGUAACTT-3'. The siRNAs were delivered at a final concentration of 1 μM using Lipofectamine® 2000 transfection reagent according to the manufacturer's instructions. The cells were incubated for 48 h to allow for MRGPRX2 knockdown.

2.11. Measurements of cytokine and protease levels

The levels of chemotactic factors in cell supernatant were detected using human chemokine array kits according to the manufacturers' instructions. After cells were incubated with SH, C48/80 or vehicle for 12 h, the levels of tumor necrosis factor (TNF)-α, interleukin (IL-8), monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein-1β (MIP-1β) were measured in modified Tyrode's solution using commercially available enzyme-linked immunosorbent assay (ELISA) kits designed for these proteins according to the manufacturers' instructions.

2.12. Western blot analysis

Total protein in untreated and SH-treated LAD2 cells (48 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 13,500g for 10 min at 4 °C. The protein concentration was determined using a BCA Protein Quantification kit accord-

ing to manufacturer's instructions. After the protein in the cell lysates was denatured by boiling the samples with a 5 × loading sample buffer from Thermo Fisher 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 from 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: anti-PLCγ1 (1:1000, #5690, Cell Signaling Technology [CST]), anti-phosphorylated-PLCγ1 (P-PLCγ1, Ser1248) (1:1000, #8713, CST), anti-IP3R (1:1000, #3763, CST), anti-phosphorylated-IP3R (P-IP3R, Ser1756) (1:1000, #8548, CST), anti-PKC (1:1000, #GR257247-4, Abcam), anti-phosphorylated PKC (P-PKC, Ser1756,) (1:500, #9371, CST), anti-P38 (1:1000, #8690, CST), anti-phosphorylated-P38 (P-P38, Thr180/Tyr182) (1:1000, #4511, CST), anti-Stim1 (1:1000, #4916, CST) or 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, after which the membranes were washed three times with TBST for 10 min and developed using an enhanced chemiluminescence (ECL) 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.

2.13. Molecular docking assay

The molecular docking was carried out using Surflex-DockMode of SYBYL-X 2.0 program package (Tripos, St. Louis, MO, USA). Thanks for Dr. Yang Zhang (Medical school, University of Michigan, USA) helped us predict the structure of MRGPRX2 by I-TASSER which is a server using for protein structure and function prediction. We used the predicted MRGPRX2 structure for docking with Sinomenine.

2.14. Statistical analysis

The group data are expressed as the mean ± S.D. Independent sample variance analysis was used to determine the significance of statistical comparisons using SPSS software. Differences were considered significant at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$).

3. Results

3.1. MrgprB2 plays an important role in SH-induced pseudo-anaphylactic reactions

A passive cutaneous anaphylaxis mouse model [25] was used to explore the *in vivo* SH-induced pseudo-anaphylactic reaction. The mice were treated with intraplantar injection of SH in the paw for 15 min, the paw thickness and Evans blue stained extravasation leakage into the paw were investigated to characterize the pseudo-anaphylactic response. The results showed that although subcutaneous injection of saline (vehicle) did not induce hindpaw inflammation (swelling and extravasation), SH injection evoked extensive extravasation and swelling in mice in a dose-dependent manner. C48/80 was used as positive control in Fig. 1A. We next tested whether the development of hindpaw inflammation after SH injection was associated with mast cells using $Kit^{W-sh/W-sh}$ mice. We observed that SH-induced hindpaw inflammation was almost com-

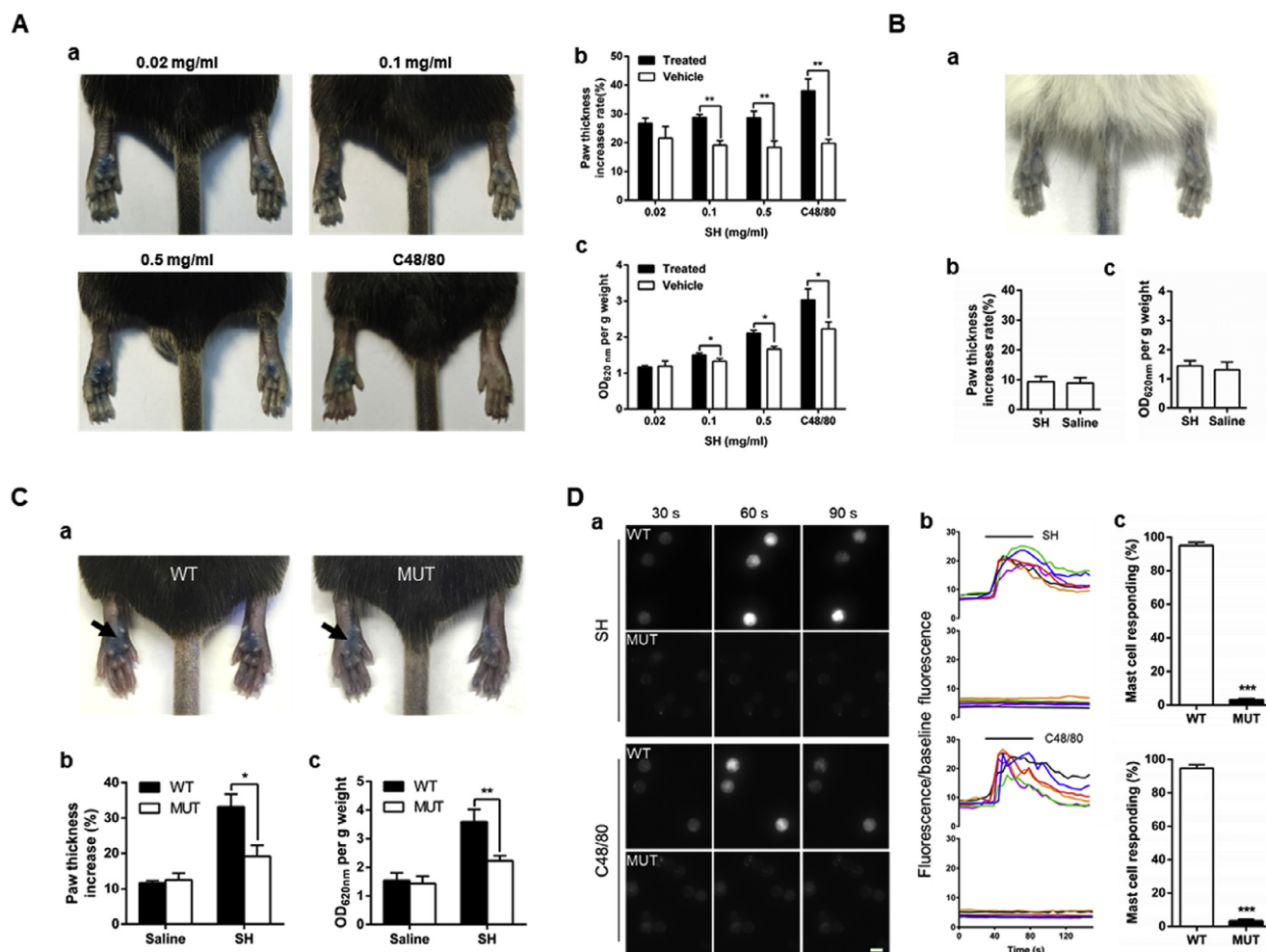


Fig. 1. SH-induced cutaneous flare reactions mediated by MrgprB2 in mast cells in mice. (A) Cutaneous flare reactions of mice treated with different concentrations of SH. (a) Representative images of Evans blue stained extravasation 15 min after intraplantar injection of 0.02 mg/ml, 0.1 mg/ml, 0.5 mg/ml SH or 10 µg/ml C48/80 in the left paw, or saline in the right paw. (b) Quantification of paw thickness increase after 15 min. (c) Quantification of Evans blue leakage into the paw after 15 min. (B) Evans blue extravasation in the paw of *Kit^{W-sh/W-sh}* mice mediated by SH. (a) Representative images of Evans blue stained extravasation 15 min after intraplantar injection of 0.5 mg/ml SH. (b) Quantification of paw thickness increase after 15 min. (c) Quantification of Evans blue leakage into the paw after 15 min. Data are presented as mean $\pm$ S.D. (C) Evans blue extravasation in the paw of MrgprB2 wild-type and MrgprB2^{-/-} (MUT) mice. (a) Representative images of Evans blue stained extravasation 15 min after intraplantar injection of 0.5 mg/ml SH. (b) Quantification of paw thickness increase after 15 min. (c) Quantification of Evans blue leakage into the paw after 15 min. Data are presented as mean $\pm$ S.D. (n = 5). (D) Calcium imaging of SH in MrgprB2 WT and MUT mouse peritoneal mast cells. (a) Representative Fluo-4 fluorescence heat map images of mouse peritoneal mast cells showing changes in $[Ca^{2+}]_i$ induced by bath application of SH (2.5 mM) or C48/80 (10 µg/ml). (b) Representative imaging traces. Each color line represents an individual cell. (c) Quantification of responding cells. Scale bar, 10 mm. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons. Statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pletely absent in C57BL/6-*Kit^{W-sh/W-sh}* mice in Fig. 1B, which indicates that the pseudo-anaphylactic reactions caused by SH were associated with mast cells. MrgprB2^{-/-} mice were used to investigate whether SH-induced hindpaw inflammation was associated with MrgprB2 expression on mast cells, and we found that compared to MrgprB2 wild type (WT) mice, MrgprB2^{-/-} (MUT) mice exhibited almost no hindpaw inflammation in Fig. 1C, which suggests that the *in vivo* activation of mast cell mediator release and the subsequent SH-induced vasodilation was related to MrgprB2.

3.2. MrgprB2 is crucial for calcium mobilization in MPMCs

Peritoneal mast cells (MPMCs) are connective tissue mast cells, which were reported expressing MrgprB2. Calcium imaging of MPMCs from WT mice showed a strongly activated response effect when treated with 2.5 mM SH, while the MUT MPMCs had almost no response (Fig. 1D), indicated a crucial role of MrgprB2 on calcium mobilization in MPMCs. C48/80 was used as a positive control. Calcium imaging of MPMCs also showed a significantly

increased intracellular Ca^{2+} release that was dose-dependent and in response to treatment with 0.5 mM, 1 mM, 5 mM or 10 mM SH and the cell responding rate were $44.41\% \pm 0.0408$, $63.08\% \pm 0.0248$, $82.96\% \pm 0.0248$ or $93.44\% \pm 0.0322$, respectively, in Fig. 2A. MrgprB2 expression has been reported to be highly specific to connective tissue mast cells, and this protein is a target of many small molecule drugs associated with treating systemic pseudo-anaphylactic and anaphylactoid reactions. Therefore, treatment with 0.5 mM, 1 mM, 5 mM or 10 mM SH in HEK293-MrgprB2 cells was implemented and showed the same trend of calcium mobilization and the cell responding rate was $20.06\% \pm 0.0027$, $49.15\% \pm 0.0271$, $91.69\% \pm 0.0647$ or $94.62\% \pm 0.0449$, respectively (Fig. 2B). The EC₅₀ of SH was 2318 ± 314 µM in HEK293-MrgprB2 cells in Table 1.

3.3. SH induce calcium mobilization in HEK293-MRGPRX2 cells

MrgprB2 is the mouse orthologue of Human MRGPRX2. Treatment with 1 µM, 5 µM, 10 µM or 100 µM SH in HEK293-

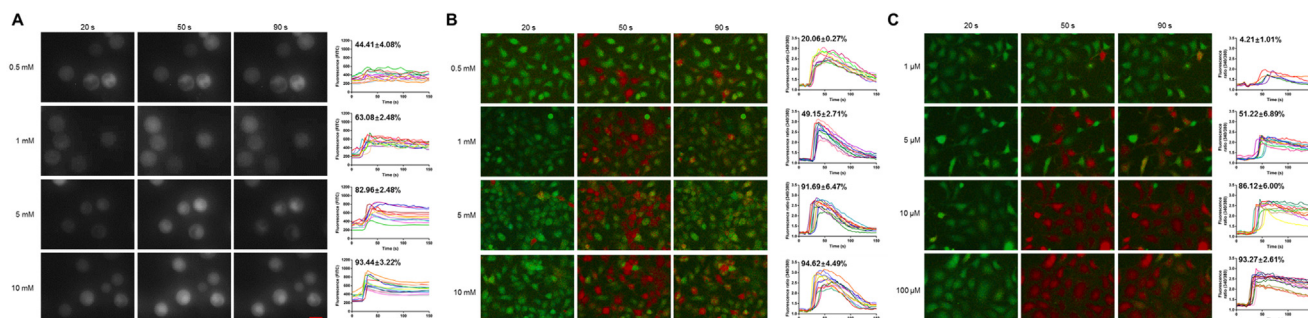


Fig. 2. Calcium imaging of SH in MPMC, HEK293-MrgprB2 and HEK293-MRGPRX2. (A) Left, representative Fluo-4 fluorescence heat map images of mouse peritoneal mast cells showing changes in $[Ca^{2+}]_i$ induced by bath application of 0.5 mM, 1 mM, 5 mM and 10 mM SH. Right, representative imaging traces and the quantification of responding cells. Each color line represents an individual cell. Scale bar, 10 μ m. (B) Left, representative Fura-2 fluorescence heat map images of in HEK293-MrgprB2 cells showing changes in $[Ca^{2+}]_i$ induced by bath application of 2 mM, 5 mM, 8 mM and 10 mM SH. Right, representative imaging traces and the quantification of responding cells. Each color line represents an individual cell. Scale bar, 20 μ m. (C) Left, representative Fura-2 fluorescence heat map images of in HEK293-MRGPRX2 cells showing changes in $[Ca^{2+}]_i$ induced by bath application of 1 μ M, 5 μ M, 10 μ M and 100 μ M SH. Right, representative imaging traces and the quantification of responding cells. Each color line represents an individual cell. Scale bar, 20 μ m. (n = 3 per concentration; 150 cells counted per condition). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

EC₅₀ of sinomenine hydrochloride in HEK293-MrgprB2 and HEK293-MRGPRX2 cells.

Substance	MrgprB2	MRGPRX2
SH	2.32 ± 0.31 mM	2.77 ± 0.44 μ M

MRGPRX2 cells showed significant dose-dependent increases in intracellular Ca^{2+} mobilization and the cell responding rate was 4.21% ± 0.0101, 51.22% ± 0.0689, 86.12% ± 0.0600 or 93.27% ± 0.0261, respectively, in Fig. 2C. SH triggered the calcium flux in lower concentrations indicated a more sensitive binding to MRGPRX2. We also found that the EC₅₀ of SH was 2.77 ± 0.44 μ M in HEK293-MRGPRX2 cells in Table 1.

3.4. MRGPRX2 mediates SH-induced degranulation of mast cells

To verify whether the SH-induced activation of mast cells promoted degranulation, we used LAD2 mast cells as a model to detect changes in the secretion of β -hexosaminidase (a degranulation marker), histamine and TNF- α , the latter two of which are mediators of increased vascular permeability. LAD2 cells were treated with different concentrations of SH (0, 50, or 100 μ M) for 30 min, after which the levels of secreted β -hexosaminidase, histamine and TNF- α were measured, 30 μ g/ml C48/80 was used as a positive control. 0, 50 μ M, 100 μ M SH or 30 μ g/ml C48/80 promoted the secretion of β -hexosaminidase into 13.21% ± 0.0128, 45.66% ± 0.0596, 52.19% ± 0.0131 or 62.55% ± 0.0169 (Fig. 3A-a) and promoted the anaphylactic mediator histamine into 44.34 ± 2.83 ng/ml, 529.44 ± 39.05 ng/ml, 566.03 ± 85.77 ng/ml or 509.92 ± 55.55 ng/ml (Fig. 3A-b), and TNF- α release was substantially increased as 3.99 ± 1.22 pg/ml, 703.41 ± 56.40 pg/ml, 723.84 ± 27.63 pg/ml or 191.04 ± 2.98 pg/ml (Fig. 3A-c) in a dose-dependent manner. Calcium imaging analysis showed that SH led to higher intracellular Ca^{2+} levels in LAD2 cells (Fig. 3A-d). MRGPRX2 knockdown LAD2 cells were performed to verify the interaction between the MRGPRX2 receptors and SH. The MRGPRX2-siRNA was used to knockdown MRGPRX2 in LAD2 cells, which showed a good knockdown efficiency (Fig. 3B). Under the treatment of 0, 50 μ M, 100 μ M SH or 30 μ g/ml C48/80, MRGPRX2 knockdown LAD2 cells showed that the levels of secreted β -hexosaminidase were 8.85% ± 0.0305, 9.02% ± 0.0031, 11.75% ± 0.0103, 30.08% ± 0.0075 (Fig. 3C-a), histamine were 48.02 ± 5.76 ng/ml, 153.08 ± 9.13 ng/ml, 108.23 ± 30.35 ng/ml, 207.16 ± 59.02 ng/ml (Fig. 3C-b), and TNF were 0.54 ± 0.08 pg/ml, 31.32 ± 0.07 pg/ml, 28.66 ± 4.68 pg/ml, 21.38 ± 0.88 pg/ml

(Fig. 3C-d). In NC transfected LAD2 cells, mediators such as β -hexosaminidase were 8.57% ± 0.0305, 21.81% ± 0.0130, 39.96% ± 0.0075, 59.49% ± 0.0528 (Fig. 3C-a), histamine released were 52.04 ± 3.27 ng/ml, 382.14 ± 6.61 ng/ml, 471.93 ± 41.60 ng/ml, 448.75 ± 180.91 ng/ml (Fig. 3C-b), and TNF- α released were 0.69 ± 0.04 pg/ml, 434.81 ± 21.32 pg/ml, 409.50 ± 21.49 pg/ml, 241.44 ± 7.51 pg/ml (Fig. 3C-d). Moreover, changes in intracellular Ca^{2+} virtually disappeared in MRGPRX2-knockdown LAD2 cells (Fig. 3C-e). Therefore, degranulation levels of MRGPRX2 knockdown LAD2 cells were significantly attenuated compared with LAD2 cells expressing MRGPRX2.

3.5. MRGPRX2 mediates SH-induced chemokines secretion in LAD2 mast cells

Chemokine secretion from mast cells were also selected LAD2 cells as the model to analyze. Treatment with 100 μ M SH on LAD2 cells for 24 h triggered significantly the expression of MCP-1, IL-8 and MIP-1 α/β expression levels based on the ELISA SPOT assays (Fig. 4A). Next, ELISA was performed to detect the secretion of MCP-1, IL-8 and MIP-1 β from cells stimulated with 0, 50 μ M, 100 μ M SH or 30 μ g/ml C48/80 as a positive control for 24 h. The concentrations of MCP-1 secreted from LAD2 mast cells were 88.35 ± 14.18 pg/ml, 1029.97 ± 91.42 pg/ml, 1067.93 ± 25.00 pg/ml, 1022.62 ± 64.05 pg/ml (Fig. 4B), respectively. The concentrations of IL-8 secreted from LAD2 mast cells were 0.99 ± 0.07 pg/ml, 94.25 ± 2.52 pg/ml, 96.59 ± 14.24 pg/ml, 82.51 ± 3.40 pg/ml (Fig. 4C). The concentrations of MIP-1 β , secreted from LAD2 mast cells were 78.70 ± 10.33 pg/ml, 1381.86 ± 339.90 pg/ml, 2501.06 ± 59.67 pg/ml, 1188.64 ± 51.25 pg/ml (Fig. 4D). Treated with SH were significantly triggered LAD2 cells secret chemokine mediators compared with the untreated control group. Furthermore, siRNA interference was used to knockdown MRGPRX2 gene expression in LAD2 mast cells. It was shown that under the treatment of 0, 50 μ M, 100 μ M SH or 30 μ g/ml C48/80, the secreted MCP-1 in NC transfected LAD2 cells were 23.90 ± 0.03 pg/ml, 1010.01 ± 46.65 pg/ml, 1021.15 ± 70.28 pg/ml, 759.55 ± 73.91 pg/ml, but in MRGPRX2 knockdown LAD2 cells were 23.14 ± 0.17 pg/ml, 162.60 ± 17.50 pg/ml, 237.51 ± 8.50 pg/ml, 68.42 ± 9.22 pg/ml (Fig. 4E), the IL-8 in NC transfected LAD2 cells were 0.91 ± 0.34 pg/ml, 192.01 ± 31.51 pg/ml, 225.90 ± 13.02 pg/ml, 93.87 ± 0.46 pg/ml, in MRGPRX2 knockdown LAD2 cells were 0.73 ± 0.01 pg/ml, 82.11 ± 20.31 pg/ml, 87.84 ± 30.91 pg/ml, 30.71 ± 5.69 pg/ml (Fig. 4F), the MIP-1 β in NC transfected LAD2 cells were 753.17 ± 10.33 pg/ml, 6109.68 ± 58.61 pg/ml, 6587.71 ± 399.44 pg/ml, 3487.41 ± 265.30 pg/ml, in

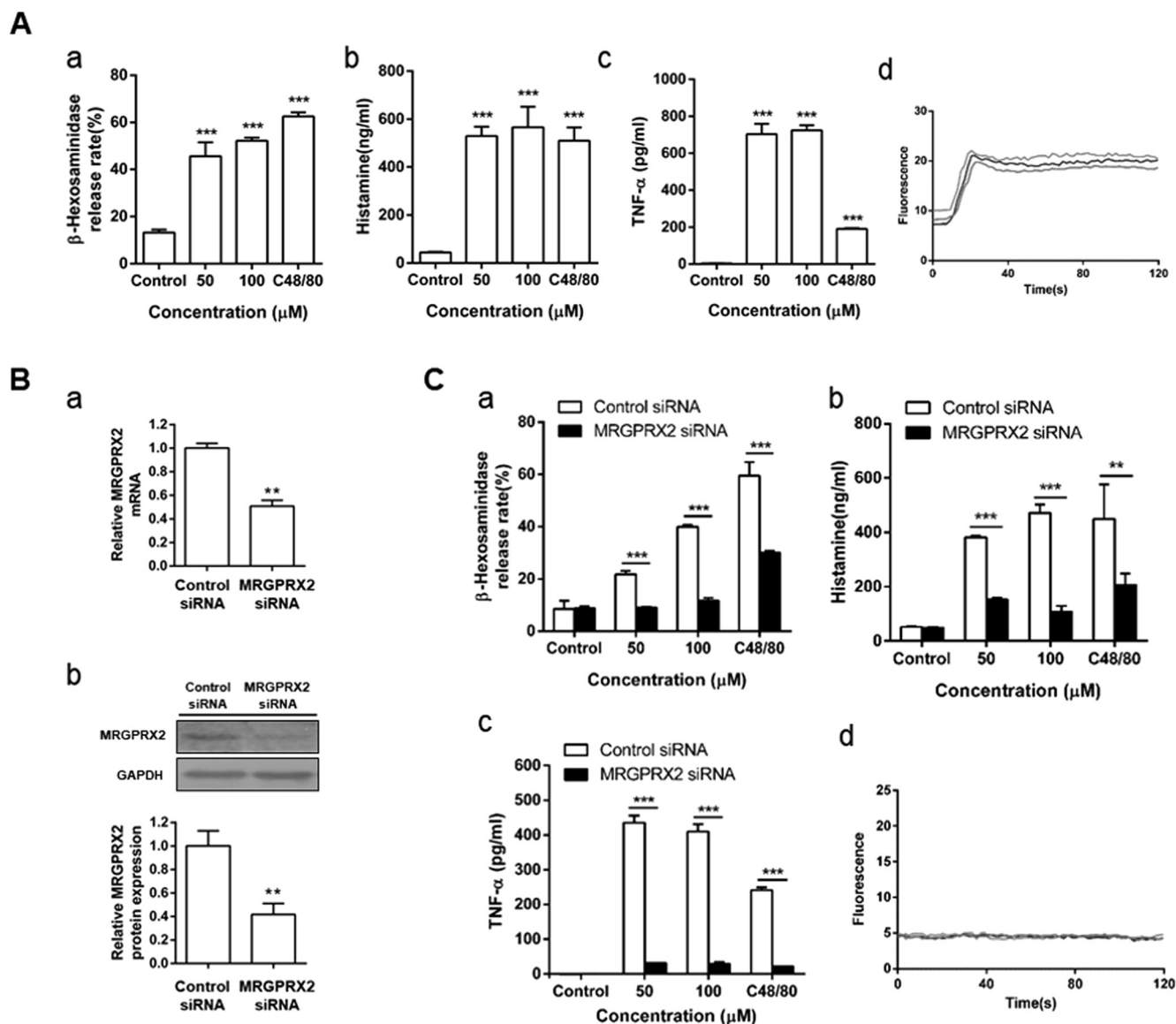


Fig. 3. MRGPRX2 plays an important role during the degranulation of mast cells triggered by SH. (A) The degranulation of LAD2 cells triggered by SH. The β -hexosaminidase (a), histamine (b) and TNF- α (c) release in LAD2 cells treated with 0, 50 μ M, 100 μ M SH or 30 μ g/ml C48/80 for 30 min. (d) Representative imaging traces Ca^{2+} concentrations treated by 100 μ M SH in negative control LAD2 cells. (B) The effect of MRGPRX2 knockdown by siRNA transfection. (a) The MRGPRX2 mRNA expression in LAD2 cells transfected with 1 μ M Negative Control siRNA (NC), MRGPRX2 siRNA respectively using Lipofectamine[®] 2000. (b) Above, protein expression levels of MRGPRX2 in the LAD2 cells transfected with siRNA. Below, Quantification of MRGPRX2 protein expression by densitometric analysis. The data are presented as mean $\pm$ S.D. (n = 3). (C) The degranulation of MRGPRX2 knockdown LAD2 cells triggered by SH. The β -hexosaminidase (a), histamine (b), TNF- α (c) release in transfected MRGPRX2 knockdown LAD2 cells treated by SH for 30 min. (d) Representative imaging traces Ca^{2+} concentrations treated by 100 μ M SH in transfected MRGPRX2 knockdown LAD2 cells. The data are presented as mean $\pm$ S.D. (n = 3). Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at *p* < .05 (**p* < .05, ***p* < .01, ****p* < .001).

MRGPRX2 knockdown LAD2 cells were 714.83 ± 41.00 pg/ml, 958.78 ± 37.74 pg/ml, 1079.89 ± 15.96 pg/ml, 1091.71 ± 17.69 pg/ml (Fig. 4G). Knockdown MRGPRX2 in LAD2 cells significantly depress the SH triggered chemokines release.

3.6. MRGPRX2 mediates SH triggered calcium mobilization during anaphylactic reactions via activating PLC- γ , IP3R, PKC and P38

Under the treatment of 10 μ M, 50 μ M and 100 μ M SH or C48/80 for 24 h, the expression of MRGPRX2 in LAD2 cells was increased in a dose-dependent manner (Fig. 5A and B), indicated that SH activated MRGPRX2 in LAD2 cells. PLC γ hydrolyzes phosphatidylinositol-4,5-bisphosphate to soluble inositol-1,4,5-trisphosphate (IP3). The binding of IP3 to IP3R causes the "first wave" of calcium mobilization, which is the transient release of Ca^{2+} from endoplasmic

reticulum stores. The results showed that when LAD2 mast cells were treated with 10 μ M, 50 μ M and 100 μ M SH for 24 h, the levels of phosphorylated PLC- γ and IP3R were increased in a dose-dependent manner (Fig. 5C and D). PKC and P38 activation are closely associated with chemokine production in mast cells. Therefore, we investigated whether SH activates PKC and P38 in LAD2 cells using Western blotting. LAD2 cells were treated with 10 μ M, 50 μ M and 100 μ M SH for 24 h, after which the levels of phosphorylated PKC (P-PKC) and phosphorylated P38 (P-P38) were determined. The results showed the SH induced P-PKC and P-P38 protein expression activities in a dose-dependent manner (Fig. 5E and F).

In order to explore the critical role of MRGPRX2 for the downstream signaling pathway, the related protein expression in MRGPRX2 knockdown LAD2 cells was tested. It was found that

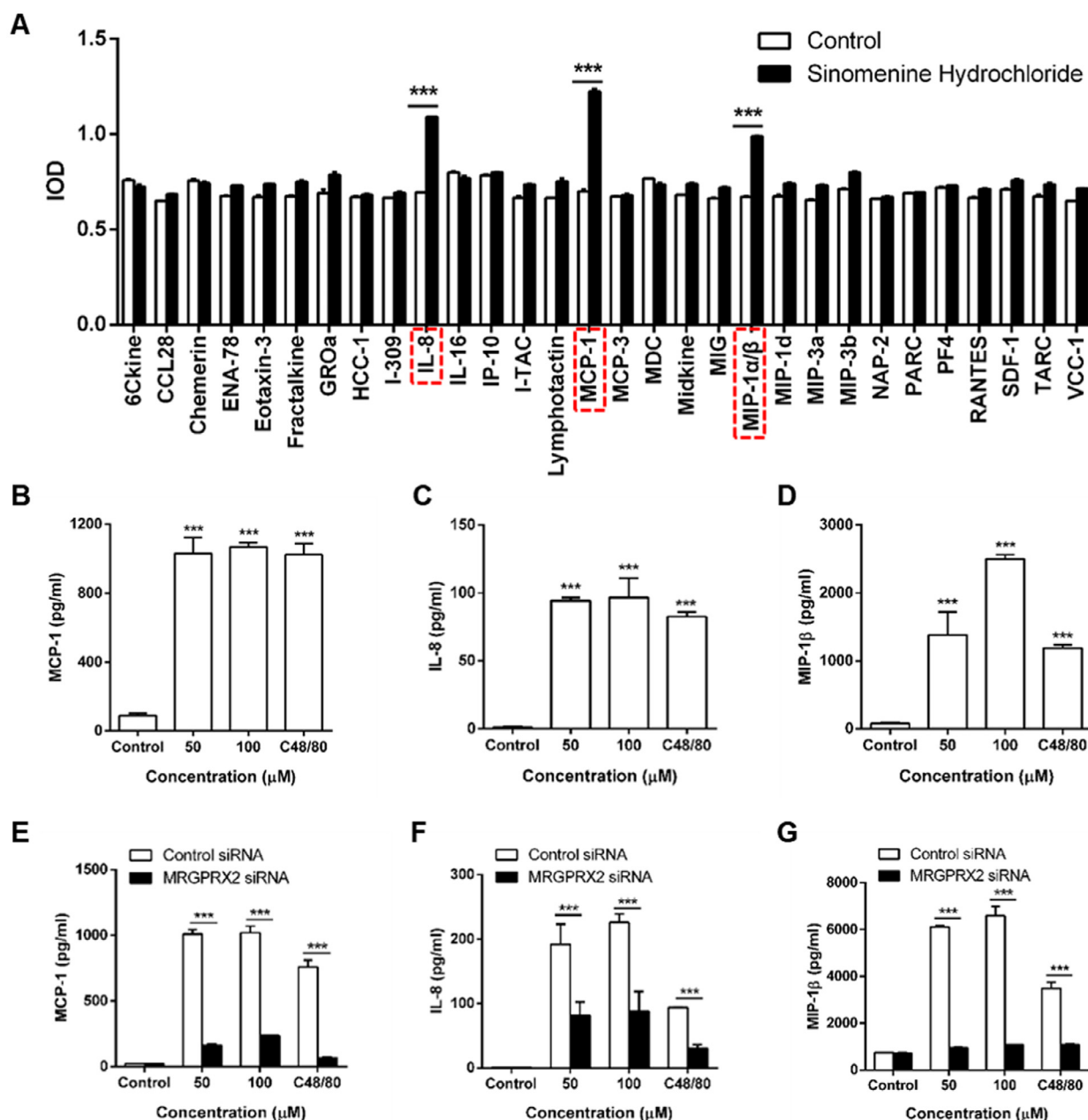


Fig. 4. Chemokines released from LAD2 mast cells activated by SH. (A) LAD2 cells were treated with SH for 24 h. Secreted cytokines were profiled using Proteome Profiler Array Mouse Cytokine array. (B, C, D) Chemokine secretions profile in LAD2 cells were detected with an ELISA array. The LAD2 cells were treated with serial concentrations of SH. (MCP-1 [B], IL-8 [C], MIP-1β [D]). (E, F, G) Chemokine secretions profile in MRGPRX2-knockdown LAD2 cells were detected with an ELISA array. (MCP-1 [E], IL-8 [F], MIP-1β [G]). The data are presented as mean $\pm$ S.D. ($n = 3$). Two-tailed unpaired Student's t -test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$).

under the treatment of 10 μ M, 50 μ M and 100 μ M SH for 24 h, the dose-dependent manner of phosphorylated PLC- γ , IP3R, PKC and P38 almost disappeared (Fig. 5G, H, I and J), which indicated that MRGPRX2 mediated the phosphorylation levels of PLC- γ , IP3R, PKC and P38 in SH activated LAD2 cells.

3.7. Stim1 was inactivated under the treatment of SH in LAD2 cells

To investigate whether SH can activate Stim1 to open the calcium channels in cell membrane, LAD2 cells were treated with 100 μ M SH for 0.5 h, 3 h, 6 h, 12 h, 24 h but showed no time-based dependency increase to the 0 h in Fig. 6 A and B. The LAD2 cells also were treated with 0, 10, 50, 100 μ M SH or 30 μ g/ml

C48/80 for 24 h and showed no dose-based dependency increased in Fig. 6C and D. We concluded that Stim1 was not involved in SH-induced LAD2 cells Ca^{2+} mobilization.

3.8. Molecular docking exhibited the activation between SH and MRGPRX2

To investigate the interactions between SH and MRGPRX2, molecular docking was carried out using Surflex-Dock Mode of SYBYL-X 2.0 program package. The docking results showed SH mach MRGPRX2 activate site well in sphere space field model in Fig. 7A, and the detailed analysis of the interaction between SH and MRGPRX2 are shown in Fig. 7B. Based on the docking results,

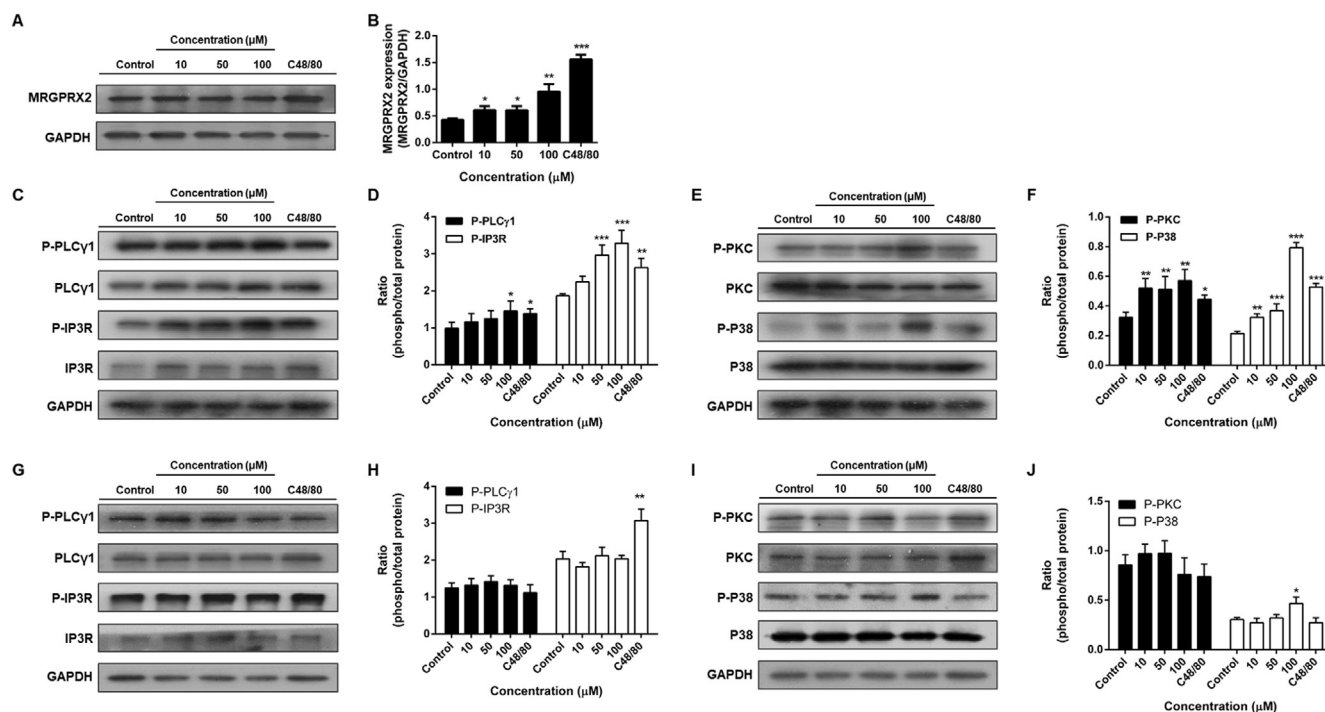


Fig. 5. Effect of SH on the protein expression of MRGPRX2 and the effect of MRGPRX2 on the protein expression of PLC- γ , IP3R, PKC and P38 in LAD2 cells. (A) Western blot analysis of the expression levels of MRGPRX2 in LAD2 cells treated with SH. (B) Quantification of MRGPRX2 protein expression in (A) by densitometric analysis. (C) Western blot analysis of the expression levels of PLC γ 1, IP3R, Phosphorylation-PLC γ 1 and Phosphorylation-IP3R in LAD2 cells treated with SH. (D) Quantification of PLC γ 1, IP3R, Phosphorylation-PLC γ 1 and Phosphorylation-IP3R protein expression in (C) by densitometric analysis. (E) Western blot analysis of the expression levels of PKC, P38, Phosphorylation-PKC and Phosphorylation-P38 in LAD2 cells treated with SH. (F) Quantification of PKC, P38, Phosphorylation-PKC and Phosphorylation-P38 protein expression in (E) by densitometric analysis. (G) Western blot analysis of the expression levels of PLC γ 1, IP3R, Phosphorylation-PLC γ 1 and Phosphorylation-IP3R in MRGPRX2 knockdown LAD2 cells treated with SH. (H) Quantification of PLC γ 1, IP3R, Phosphorylation-PLC γ 1 and Phosphorylation-IP3R protein expression in (G) by densitometric analysis. (I) Western blot analysis of the expression levels of PKC, P38, Phosphorylation-PKC and Phosphorylation-P38 in MRGPRX2 knockdown LAD2 cells treated with SH. (J) Quantification of PKC, P38, Phosphorylation-PKC and Phosphorylation-P38 protein expression in (I) by densitometric analysis. The data are presented as mean $\pm$ S.D. (n = 3). Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$).

SH formed one hydrogen bond with TRP179 and the length was 2.36 Å.

4. Discussion

The pathogenesis of an anaphylactoid reaction, which is a non-IgE-mediated anaphylactic reaction, could be caused by directly activating anaphylactic inflammatory effector cells or mast cells by certain substances (excluding IgE, IgG, or T-cell mediation) [18]. Mast cells are a class of human immune cells that are primarily distributed in body parts exposed to the external environment such as the skin, lungs, and gastrointestinal tract [26]. It plays an important role in the immune regulation of the tissue microenvironment [27] and participate in various disorders and physiological processes such as drug induced anaphylactic reactions, asthma, hypertrophy, and histamine release (a small molecule) [28]. Therefore, we investigate whether SH (main substance in ZQFTN preparation) could interact with MRGPRX2 and induce mast cells degranulation.

LAD2 cells are skin-derived mast cells found in human connective tissue. Mature LAD2 cells express high levels of MRGPRX2 which is an ideal model for studying anaphylactoid reactions [29]. Recently, studies have reported that opioid receptor compounds such as morphine and SH can activate MRGPRX2 and cause LAD2 cell degranulation [24]. In accordance with this, we observed that SH causes a dose-dependent increase in the concentration of intracellular calcium ions in LAD2 mast cells, which triggers degranulation, and that this activity is mediated by MRGPRX2. However, the EC₅₀ analysis showed significant differences in the

intensity of SH stimulation at two receptor subtypes, which is consistent with the earlier report by McNeil [22]. Based on the predicted MRGPRX2 tertiary structure, the molecular docking results showed a good structure-activity relationship with SH. Previous study found that SH induce RBL-2H3 cells and P815 basophils cell degranulation in a dose-dependent manner [20,21]. The receptor triggering mast cell degranulation remains unclear. Therefore, it is important to investigate mechanism of SH degranulation effect.

Activation of mast cells result in the release of various bioactive substances. A classic anaphylactic reaction can manifest due to the binding of specific IgE with high-affinity IgE receptors (Fc ϵ RI) on the surface of mast cells causing the release of various bioactive substances (e.g., histamine, heparin, tryptase, slow reacting substances, chemokines, and other inflammatory mediators [30] and the consequent impact of these mediators on effector tissues or/and organs. In 2015, Grimaldeston discovered that there is a difference between the MRGPRX2- and IgE-Fc ϵ RI-induced mast cell degranulation signaling pathways [31]. In this study, we found that SH regulates intracellular calcium fluctuations via MRGPRX2-PKC signaling and induces mast cell degranulation. The prolonged stimulation could phosphorylate PKC and P38 thereby promoting the synthesis of pro-inflammatory cytokines, which elicits an effect to that of IgE receptor activity. It is well known that IgE-Fc ϵ RI can induce intense and prolonged degranulation due to its induction of large-scale synthesis of pro-inflammatory factors [32]. Corroborating with the findings, we observed that the synthesis of pro-inflammatory cytokines requires prolonged stimulation by high-concentration stimulus.

This study indicates that the interaction between MRGPRX2 and SH caused PLC γ 1 and IP3R phosphorylation in mast cells. Certain

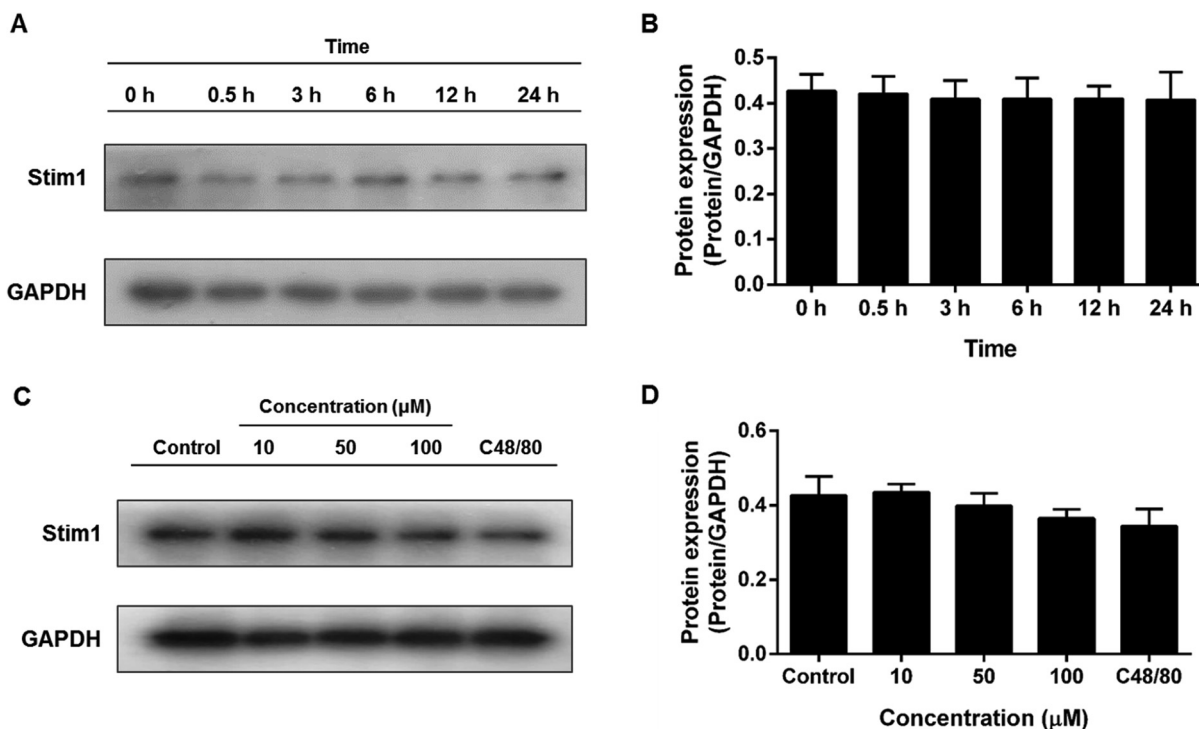


Fig. 6. Effect of SH on the expression of Stim1 in LAD2 cells. (A) Western blot analysis of the expression levels of MRGPRX2 in LAD2 cells treated with SH for 0 h, 0.5 h, 3 h, 6 h, 12 h or 24 h. (B) Quantification of MRGPRX2 protein expression by densitometric analysis. (C) Western blot analysis of the expression levels of MRGPRX2 in LAD2 cells treated with 10 μM, 50 μM, 100 μM SH or 10 μg/ml C48/80 for 24 h. (D) Quantification of MRGPRX2 protein expression by densitometric analysis. The data are presented as mean ± S.D. (n = 3). Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at *p* < .05.

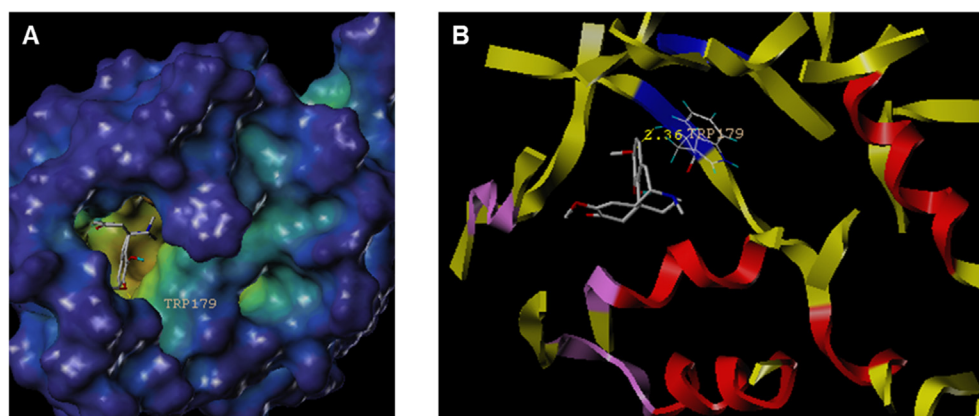


Fig. 7. Molecular docking modeling of sinomenine with MRGPRX2. (A) Sphere space field model of sinomenine and MRGPRX2. (B) Ribbon model of sinomenine and MRGPRX2.

drugs that trigger mast cell degranulation directly promote diacylglycerol (DAG) and inositol triphosphate (IP₃) synthesis via the MRGPRX2-PLC pathway, which causes the release of calcium ions from the endoplasmic reticulum (ER) [33,34]. Consequently, the calcium sensor Stim1 on the ER interacts with the membrane protein Orai1 [35], thereby opening the calcium ion channel CRAC. This leads to an additional increase in the levels of intracellular calcium ions, which then induces cytoskeletal reorganization and subsequent degranulation. However, we did not observe Stim1 increase in SH activated mast cells. The SH-induced intracellular calcium ion influx was primarily due to the release of calcium ions from the ER in the mast cells.

Mast cells can respond to basic secretagogues, including mastoparan, Substance P and C48/80, as well as some peptidergic drugs

associated with adverse reactions [36–38], SH may have the same response to mast cells. Those peptidergic drugs are thought to activate pertussis toxin-sensitive G proteins (for example, G_{i2} and G_{i3}) in a receptor independent manner on the mast cell surface [39], which was proved to be MRGPRX2 [22], and the signaling pathways include phospholipase C-γ, and phosphatidylinositol 3-kinase, which then promote calcium mobilization that leads to exocytosis of preformed granule-associated mediators (such as, histamine) [39]. Like this, SH active MRGPRX2 and may propagate signaling via these pathways. However, the specific molecular mechanisms underlying SH-induced activation of MRGPRX2 have been poorly understood until now.

In conclusion, this study demonstrated that SH induces anaphylactoid reactions by activating mast cells to undergo degranulation,

which releases mediators such as β -hexosaminidase, histamine, and TNF- α as well as chemokines such as MCP-1, IL-8, and MIP-1 β . As an opioid receptor agent, SH can upregulate PLC expression via MRGPRX2 signaling pathways to promote calcium ion release from the ER, which is key step to induce mast cell degranulation. This study systematically elucidated the SH-mediated effects and molecular mechanism on drug-induced pseudo-allergic reactions, which lays a foundation for the pre-clinical development of drugs using SH as an initial candidate compound.

Conflict of interest statement

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

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Number: 81230079 and 81227802), the Fundamental Research Funds for the Central University (Grant Number: xjj2016125) and foundation from Shaanxi Administration of Traditional Chinese Medicine (Grant Number: 15-ZY032).

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