

RESEARCH ARTICLE

The anti-anaphylactoid effects of Piperine through regulating MAS-related G protein-coupled receptor X2 activation

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Mast cells play an important role in inflammatory and allergic diseases. MAS-related G protein-coupled receptor X2 (MRGPRX2) is a novel G protein-coupled receptor in mast cells that mediates drug-induced anaphylactoid reactions. Piperine has been reported to have anti-inflammatory and anti-allergic pharmacological activities. However, whether the pharmacological effects are regulated by MRGPRX2 has not yet been reported. The purpose of this study was to assess the anti-anaphylactoid effect of Piperine and to explore its potential mechanism. The anti-anaphylactoid effect of Piperine was assessed by an in vivo mouse hindpaw extravasation model. Mast cell intracellular calcium mobilization was measured by a calcium imaging assay. An enzyme immunoassay was used to evaluate the release of pro-inflammatory factors from stimulated mast cells. Activated mast cell related signals were assessed by western blot. A cell membrane chromatography assay was used to determine the binding characteristics of Piperine and MRGPRX2. The results showed that Piperine suppressed mast cell intracellular Ca^{2+} mobilization, inhibited cytokines and chemokines release, and down-regulated the phosphorylation level of phospholipase $\text{C}\gamma 1$, protein kinase C, inositol 1,4,5-triphosphate receptor, P38, protein kinase B, and ERK. Meanwhile, Piperine can bind to MRGPRX2 as a specific antagonist. Hence, Piperine can be served as a novel therapeutic drug candidate for MRGPRX2-mediated anaphylactoid reactions.

KEYWORDS

antianaphylactoid effects, Ca^{2+} influx, mast cells, MRGPRX2, Piperine

1 | INTRODUCTION

Anaphylactoid reaction is a well-recognized and life-threatening adverse drug reaction. The usual clinical manifestations include pruritus, angioedema, urticaria, bronchospasm and wheezing, hypotension,

vomiting, nausea, and diarrhea (Castells, 2017; Ellis & Day, 2003). In severe cases, it may lead to upper respiratory complications, such as suffocation, hypotension, or cardiovascular failure, and would be life-threatening (Lieberman et al., 2010). Anaphylactoid or pseudo-allergic reaction was usually caused by mast cells and basophils degranulation, due to the direct stimulation by many kinds of drugs, such as quinolones, radioactive contrast agents, lipophilic micellar solvents, narcotic drugs, analgesics, and hemostatic agents (Jackman et al., 2016; Szebeni, 2014), leading to mast cells degranulation (Aun et al., 2014; Lieberman & Garvey, 2016; McNeil et al., 2015). These mediators comprise histamine, neutral proteases, proteoglycans, products of

Abbreviations: Akt, protein kinase B; CMC, cell membrane chromatography; C48/80, compound 48/80; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular-signal regulated kinases 1/2; IL, interleukin; IP3R, 1,4,5-triphosphate receptor; LAD2, laboratory of allergic disease 2; MCP, monocyte chemoattractant protein; MPMCs, mouse peritoneal mast cells; MRGPRX2, MAS-related G protein-coupled receptor X2; P-, antiphosphorylated-; PLC $\gamma 1$, phospholipase $\text{C}\gamma 1$; PKC, protein kinase C; TNF- α , tumor necrosis factor- α .

arachidonic acid metabolism, and some other cytokines and chemokines (Gill, Jindal, Jagdis, & Vadas, 2015). The symptoms are related to the dosage of agents, and results in fast and high content release of bioactive substances, and may lead to a more severe allergic symptoms (Meng, Ariza, Waddington, Park, & Naisbitt, 2016).

Mast cells activation is crucial for allergic and inflammatory diseases (Bulfone-Paus, Nilsson, Draber, Blank, & Levi-Schaffer, 2017). The calcium motivation of mast cells induces the activation of downstream protein signaling molecules. Upon stimulation, the inositol 1,4,5-triphosphate receptor (IP3R) is activated, and Ca^{2+} is released from the endoplasmic reticulum into the cytoplasm, which will induce the protein kinase C (PKC) activation (Sibilano, Frossi, & Pucillo, 2014). The activated PKC will phosphorylate the downstream related mitogen-activated protein kinase signaling molecules. Phospholipase C (PLC) is one of the regulatory factors of IP3R, which is also regulated by G proteins (Solinski, Gudermann, & Breit, 2014).

Recently, MAS-related G protein-coupled receptor-X2 (MRGPRX2) was reported to affect immunoglobulin E-independent anaphylaxis induced by some specific drugs, such as non-beta-lactam antibiotics, steroidal antiinflammatory drugs, quinolones, opioids, and vancomycin (Chen et al., 2018; McNeil et al., 2015; Montanez et al., 2017; Subramanian, Gupta, & Ali, 2016a). Therefore, MRGPRX2 is crucial during certain drug-induced anaphylactoid reactions.

At present, the treatment of allergic-like diseases is mainly to recovery immune disorders and attenuate allergy related inflammatory release. Anti-allergy drugs mainly include anti-histamines, such as Loratadine and Cetirizine; mast cell stabilizers, such as Crdmolyn Sodium and Ketotifen; and hormone drugs, such as Dexamethasone and Cyclophosphamide (Church, 2017; Joks & Durkin, 2011). However, it is important to find more effective candidate drugs to suppress mast cell activation during MRGPRX2-dependent anaphylactoid reactions.

Piperine (1-(5-[1,3-benzodioxol-5-yl]-1-oxo-2,4-pentadienyl) piperidine; Figure 1) is a kind of pungent nitrogenous alkaloid that is present in the fruit of long pepper (*Piper longum* L.), black pepper (*Piper nigrum* L.) and other *Piper* species (Gorgani, Mohammadi, Najafpour, & Nikzad, 2017; Meghwal & Goswami, 2013; Srinivasan, 2007). It was reported that Piperine has exhibited anti-inflammatory,

anti-oxidant, hepatoprotective, and antiulcer activities in preclinical studies (Chavarria, Silva, Magalhaes e Silva, Remiao, & Borges, 2016; Srinivasan, 2007). Currently, more and more studies have reported about the anti-anaphylactoid compounds that are from natural products, which indicated potential antagonists of MRGPRX2 (Ding et al., 2019; Hou et al., 2018; Wang et al., 2018).

Hence, we devoted to studying the antianaphylactoid effects of Piperine and the crucial role of MRGPRX2 during the process. The antianaphylactoid effects were performed by a compound 48/80 (C48/80)-induced mice hindpaw cutaneous flare assay. Human laboratory of allergic disease 2 (LAD2) mast cells and mouse peritoneal mast cells (MPMCs) were used to investigate the inhibitory effect of Piperine via the calcium imaging assay and the degranulation detection. The mast cell activation related signaling were also be studied by the western blotting assay. A cell membrane chromatography (CMC) assay was applied for binding characteristics of Piperine with MRGPRX2.

2 | MATERIALS AND METHODS

2.1 | Drugs and reagents

Piperine (CAS No. 94-62-2) with a purity of 98% was purchased from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, Sichuan, China). Dimethyl sulfoxide, C48/80 (a classic mast cell activator and canonical basic secretagogue), *p*-nitrophenyl *N*-acetyl- β -D-glucosamide, and Evans blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chromatographic methanol (Cat. No. M116118) was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Saline was purchased from Shandong Qidu Pharmaceutical Co., Ltd. (Jinan, Shandong, China). Annexin V-FITC/PI Apoptosis Detection Kit (Cat. No. A005-3) was purchased from 7sea Biotech Co., Ltd. (Shanghai, China). The Human Enzyme-linked Immunosorbent Assay (ELISA) Kits of Interleukin (IL)-8 (Cat. No. EH005-96), tumor necrosis factor (TNF)- α (Cat. No. EH009-96) and monocyte chemotactic protein (MCP)-1 (Cat. No. EH019-96) were purchased from ExCell Biology, Inc. (Shanghai, China). The prostaglandin D_2 -MOX Express ELISA Kit (Cat. No. 500151) was purchased from Cayman Chemical Company Inc. The IL-31 ELISA Kit (Cat. No. E-EL-H5469c) was purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, Hubei, China). StemPro-34 medium (Cat. No. 10640-019), StemPro nutrient supplement (Cat. No. 10641-025), L-glutamine (Cat. No. 21051024) were obtained from Gibco (Logan, UT, USA). Penicillin-streptomycin solution (10x) was purchased from Xi'an Hat Biotechnology Co., Ltd. (Xi'an, Shaanxi, China). Recombinant human stem cell factor (Cat. No. TL-504) and recombinant mouse stem cell factor (Cat. No. JN0249-DNG) were obtained from Sino Biological Inc. (Beijing, China). Dulbecco's Modification of Eagle's Medium high glucose (Cat. No. SH30022.01) and fetal bovine serum (Cat. No. 16140071) were obtained from HyClone (Logan, UT, USA). Fluo-3 AM was procured from Thermo Fisher Scientific (Waltham, MA, USA). Pluronic F-127 gel was procured from Biotium (Fremont, CA, USA). The protease

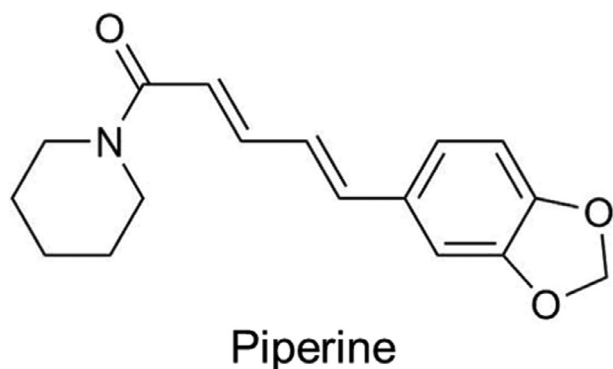


FIGURE 1 Chemical structure of Piperine

inhibitor and phosphatase inhibitor cocktails were obtained from Roche Diagnostics (Shanghai) Ltd. (Shanghai, China). Pentobarbital sodium was procured from Xiya (Jinan, Shandong, China).

2.2 | Drug administration in vitro and in vivo

A 8×10^{-2} M stock of Piperine dissolved in dimethyl sulfoxide was prepared for the in vitro assay, and diluted in control buffer. Piperine was dissolved in 0.3% CMC-Na buffer at a dosage of 0.1 mL per 10 g (body weight) mice for in vivo administration. 0.3% CMC-Na buffer was used in the control group. The administration timings and routes were mentioned among the corresponding experimental description. C48/80 is a kind of basic secretagogue, which can directly promote mast cell degranulation via MRGPRX2 rather than high-affinity immunoglobulin E (McNeil et al., 2015). Therefore, the anti-anaphylactoid effects of Piperine can be proved by its prevention ability to C48/80-induced mast cell degranulation. The concentrations of Piperine used in the experiments were according to Huang's paper (Huang et al., 2014) and the preliminary experimental results.

2.3 | Cell lines

LAD2 human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe in the National Institutes of Health (Bethesda, MD, USA). These cells were cultured in StemPro-34 medium supplemented with 2 mM L-glutamine, StemPro-34 nutrient supplement, 100 µg/mL Streptomycin, 100 Units/mL Penicillin, and 100 ng/mL recombinant human stem cell factor and cultured in the incubator of 5% CO₂ at 37°C. Replace the culture medium every 7 days, cell density was maintained less than 2×10^6 cells/mL. MRGPRX2/HEK 293 cells were kindly provided by Professor Xinzhong Dong from Johns Hopkins University (Baltimore, MD, USA), cultured in Dulbecco's Modification of Eagle's Medium.

2.4 | Mouse model

C57BL/6 mice were purchased from Xi'an Jiaotong University Experimental Animal Center (Xi'an, Shaanxi, China). Six- to eight-week-old adult male mice were used in this study. All the experiments in mice were conducted by randomized, double-blind study in the conditions. All the mouse models were housed in the experimental area the day before experiments.

2.5 | Ethics statement

The mice experimental protocols were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit No. XJTULAC2019-1019).

2.6 | Hindpaw swelling and extravasation assay

Randomized groups of mice were treated with 0-, 2-, 10-, or 20-mg/kg Piperine in 3% CMC-Na orally to investigate the anti-swelling effect of Piperine; 3% CMC-Na buffer was used as control. The mice were anesthetized with 50 mg/kg pentobarbital sodium by intraperitoneal injection after 30 min. Fifteen minutes after anesthesia, each mouse was intravenously injected with 50 µL of 12.5% Evans blue in saline. 5 µL of 30 µg/mL C48/80 were subcutaneously injected in one hindpaw of each mouse and 5 µL saline in the other hindpaw as a negative control. A Vernier caliper was used to measure the thickness of the hindpaw before and 15 min after the subcutaneous injection. Mice were then euthanasia by excessive CO₂ inhalation, and the hindpaw tissues were collected and dried at 50°C and subsequently weighed individually. Next, Evans blue dye of each hindpaw was extracted from the tissues by the acetone-saline buffer (7:3) at the volume of 500 µL. Two hundred microliters of the supernatant solution was equally distributed into 96 well plates and read the optical density value at 620 nm by a spectrophotometer.

2.7 | Body temperature

The anal temperature of mice was measured by a Model FT3400 animal temperature tester (Nanjing Calvin biotechnology Co. Ltd., Nanjing, Jiangsu, China). Divide the mice randomly into two groups ($n = 5$ for each group), and treat them with 20 mg/kg Piperine in CMC-Na orally; the same volume CMC-Na was used in control group. The mice were then injected intravenously with 200 µL of 10 µg/mL C48/80. Record the body temperature by using the biological function experimental system, and a probe was inserted into the anus to get the rectal temperatures, the temperature was measured every 3 min and continued for a total of 30 min.

2.8 | Mouse peritoneal mast cells purification

Each mouse was euthanized by inhalation excessive CO₂. A total of 12 mL ice-cold mast cell dissociation media (MCDM, Hanks' Balanced Salt Solution with 10 mM HEPES and 3% fetal bovine serum, pH 7.2) was used to obtain two to three sequential peritoneal lavages, then collected the medium together and centrifuged at 200 g and 4°C for 5 min. The cells were resuspended in the bottom by 2 mL MCDM, layered over 4 mL isotonic 70% Percoll suspension (2.8 mL Percoll, 40 µL of 1 M HEPES, 830 µL mast cell dissociation media, and 320 µL 10× Hanks' Balanced Salt Solution) and centrifuged at 500 g and 4°C for 20 min. The pellet was mast cells, washed them with Hanks' Balanced Salt Solution twice, and the purity was confirmed at >95% by toluidine blue staining and morphological analysis, which was shown in the previous study (Liu et al., 2017). The mast cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) with 25-ng/mL recombinant mouse SCF at a density of 5×10^5 to 1×10^6 cells/mL for 2 hr before experiments.

2.9 | Intracellular Ca^{2+} mobilization assay

MPMCs were washed twice with 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, adjusted to pH 7.4 with NaOH). And then MPMC cells were incubated with 0, 50, 100, or 200 μM Piperine diluted in consisted of 4 μM Fluo-3 AM calcium imaging buffer for 30 min. Cells were washed twice with calcium imaging buffer, and were imaged with excitation at 488 nm. For imaging, 30 $\mu\text{g}/\text{mL}$ of C48/80 was used to triggered each group cells beginning from 10 s, and the responses were monitored continuously for 120 s.

2.10 | Cell viability assay

The cytotoxicity effects of Piperine on LAD2 cells were assessed using the Annexin V-FITC/PI Apoptosis Detection Kit. Briefly, 2 mL LAD2 cells were seeded at a density of 5×10^4 cells/well in the 96-well plates, and treated with Piperine subsequently at 12.5, 25, 50, 100, 200, or 400 μM for 24 h. After the treatment, LAD2 cells were incubated with the working solution in accordance with the manufacturer's instruction and were analyzed by a flow cytometry.

2.11 | β -hexosaminidase release assay

Two by 10^4 cells/well LAD2 cells were seeded in a 96-well plate for 2 hr. After centrifugation at 200 g for 5 min, the supernatant was discarded and 100 μL of mast cell agonists were added, such as, 30 $\mu\text{g}/\text{mL}$ C48/80, 30 $\mu\text{g}/\text{mL}$ LL-37, or 200 μM Ciprofloxacin, contained 0, 50, 100, or 200 μM Piperine diluted in Tyrode's solution (120 mM NaCl, 2.5 mM CaCl_2 , 4.7 mM KCl, 1.2 mM MgSO_4 , 10 mM HEPES, 1.2 mM KH_2PO_4 , 5.5 mM glucose, and 5 mM bovine serum albumin), and then incubated for 30 min. For this experiment, β -hexosaminidase released into the supernatant, the total β -hexosaminidase content of mast cells was lysed with 0.1% Triton X-100. The β -hexosaminidase could be hydrolyzed by *p*-nitrophenyl *N*-acetyl- β -D-glucosamide (dissolved in 0.1 M citric acid/sodium citrate buffer, pH 4.5), and the hydrolysis would be quantified by the carbonate/sodium bicarbonate (0.1 M, pH 11.0) and measured at 405-nm absorption wavelength using a microplate reader. Mast cell agonists with no Piperine was used as control.

2.12 | Histamine release assay

Histamine 2HCl (A, A, B, B-D4, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). High performance liquid chromatography-grade acetonitrile and methanol were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Mass spectrometry-grade formic acid and histamine was obtained from Sigma-Aldrich (St. Louis, MO, USA). A LCMS-8040 mass spectrometer was purchased from Shimadzu Corporation (Kyoto, Japan). A

hydrophilic interaction liquid chromatography column, 2.1 mm $\times$ 150 mm, 3 μm , was purchased from Agela Technologies (Tianjin, China). Histamine content was evaluated on the system using the hydrophilic interaction liquid chromatography column and an isocratic elution with mixed solution of acetonitrile–water added with 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 mL/min. Two by 10^4 cells/well LAD2 cells were seeded in the 96-well plate, replaced the culture medium with 100 μL of Tyrode's solution that contained 50, 100, or 200 μM Piperine and mast cell agonists, and then incubated for 30 min. The plate was centrifuged at 200 g for 5 min. The cells supernatant were collected for histamine measuring. Mast cell agonists with no Piperine was used as control.

2.13 | Measurements of cytokines

Two by 10^4 cells/well LAD2 cells in a 96-well plate were incubated with 100 μL of 0, 50, 100, or 200 μM Piperine that contained 30 $\mu\text{g}/\text{mL}$ C48/80 for 6 hr; the levels of prostaglandin D_2 , TNF- α , IL-31, IL-8 and MCP-1 were measured using commercially available ELISA kits according to the manufacturers' instructions.

2.14 | Western blot analysis

The tested protein was extracted from LAD2 cells treated with 0, 50, 100, and 200 Piperine that contained 30 $\mu\text{g}/\text{mL}$ C48/80 for 30 min using a radioimmunoprecipitation assay lysis buffer containing 10% phosphatase inhibitor and protease inhibitor cocktails for 30 min on ice. After a centrifugation at 12,000 g for 10 min at 4°C, the protein lysate was cleared. A bicinchoninic acid protein quantification kit was used to determine the protein concentration. A 5 $\times$ loading sample buffer was added to the cell lysates and were denatured by boiling for 5 min. The denatured proteins were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The polyvinylidene fluoride membranes was used for proteins to transfer and then blocked the transferred proteins with 5% nonfat milk for 2 hr. The proteins in the membranes were then incubated with specific primary antibodies, including anti-GAPDH (1:2000, #2118, Cell Signaling Technology [CST]), antiphosphorylated-phospholipase $\text{C}\gamma 1$ (P-PLC $\gamma 1$, Ser1248; 1:1000, #8713, CST), anti-phospholipase $\text{C}\gamma 1$ (PLC $\gamma 1$; 1:1000, #5690, CST), antiphosphorylated-IP3R (P-IP3R; Ser1756; 1:1000, #8548, CST), anti-IP3R (1:1000, #3763, CST), antiphosphorylated PKC (P-PKC; Ser1756; 1:500, #9371, CST), anti-PKC (1:1000, #GR257247-4 Abcam), antiphosphorylated-Akt (P-Akt; Ser473; 1:1000, #4060, CST), anti-Akt (protein kinase B; 1:1000, #4691, CST), antiphosphorylated-extracellular-signal regulated kinases (ERK)1/2 (P-ERK1/2, P-p44/42 mitogen-activated protein kinase [ERK1/2] [Thr202/Tyr204], 1:1000, #9101, CST), anti-ERK1/2 (1:1000, p44/42 mitogen-activated protein kinase [ERK1/2], #9102, CST), antiphosphorylated-P38 (P-P38, Thr180/Tyr182; 1:1000, #4511, CST), or anti-P38 (1:1000, #8690, CST) antibodies

overnight at 4°C, and then washed three times every 10 min by TBST, and followed by incubated with secondary antibodies for 1 hr at 37°C. After being washed, the membranes were developed using an excellent chemiluminescent substrate kit, and then scanned the film and quantified the protein levels by Image-Pro Plus 5.1 software.

2.15 | The measurement of K_D value of Piperine binding to MRGPRX2 by CMC

CMC method is an effective technique to study the characteristics of drug-membrane receptor affinity, which can be used to determine the K_D value of drug-membrane receptor (Ma et al., 2017; Ma, Yang, & He, 2018). For this experiment, CMC analysis was performed on a Shimadzu LC-20A apparatus that consisted of two LC-20AD pumps, a DGU-20A3 degasser, a SIL-20A auto sampler, a CTO-20A column oven, and a SPD-M20A diode array detector (Shimadzu, Kyoto, Japan). The data were acquired by the LC solution software (Shimadzu, Kyoto, Japan). Five hundred micromolar of sinomenine hydrochloride, the known agonist for MRGPRX2 (Lansu et al., 2017; Liu et al., 2017), were prepared by dissolving the standard drugs in methanol. Exponentially growing MRGPRX2/human embryonic kidney 293 cells were harvested using trypsin and washed three times with saline by centrifuging at 500 g for 5 min, and the pellet was resuspended with 50 mM Tris-HCl (pH 7.4), followed by ultrasonic destruction for 30 min. The cell membrane was harvested in the supernatant by centrifugation at 1,000 g for 10 min and then centrifuged the supernatant at 12,000 g for 10 min. The precipitate was then suspended with saline. Briefly, the MRGPRX2 cell membrane immobile phase was prepared by adsorption of the cell membrane suspension (5 mL) on the 105°C activated silica (0.05 g) under vacuum and with a gentle agitation. The MRGPRX2 cell membrane immobile phase was placed overnight and then washed with saline three times. Finally, the mixture obtained was packed into a column (10 × 2.0-mm inner diameter) using a wet packing method (10 MPa, 5 min). All the procedure was performed at 4°C.

In the frontal analysis studies, the CMC column was first equilibrated with water as the mobile phase buffer. The mobile phase was then switched to water that contained a known concentration of Piperine in the same liquid. These solutions included up to six concentrations that all ranged from 0 to 3.2×10^{-7} M. 500 μ M of sinomenine hydrochloride was applied to the column under each Piperine solution was used as mobile phase.

In the CMC model, the K_D value is investigated by using frontal analysis and the relative K_D value as the following equation (Ma et al., 2017; Ma et al., 2018):

$$\frac{1}{k} = \frac{K_{DA}V_M}{K_{DI}m_L}[A] + \frac{K_{DA}V_M}{m_L}, \quad (1)$$

where k is the capacity factor of the applied analyte, K_{DA} is the equilibrium dissociation constant of the known analyte, K_{DI} is the

dissociation constant of the applied analyte, $[A]$ is the molar concentration of applied analyte, m_L is the total moles of binding sites in the column, V_M is the dead volume of the CMC. In the CMC results, the k value could be calculated by the equation, $k = (t_R - t_0)/t_0$, where t_0 is the retention time of solvent, and t_R is the retention time of the known analyte. For the calculation of the applied analyte, a plot of $1/k$ versus $[A]$ should produce a linear relationship for a system, where the value of K_{DI} can be found by taking the ratio of the intercept over the slope (Ma et al., 2018). The experiment was repeated for three times, and the data represents the means $\pm$ standard error of the mean from three repeated experiments.

2.16 | Statistical analysis

The significance of statistical comparison was determined using independent sample analysis of variance by SPSS software. Data analysis was performed using the Mann-Whitney test or Kruskal-Wallis test. Individual differences between the treatment and control groups were determined using Dunn's test. Statistical significance was considered at $p < .05$ (* $p < .05$, ** $p < .01$, and *** $p < .001$).

3 | RESULTS

3.1 | Piperine attenuated C48/80-induced cutaneous flare in mice

To confirm whether Piperine could decrease C48/80-induced skin inflammation in vivo, a C48/80-induced cutaneous flare model was generated to evaluate the impact of Piperine on hindpaw edema in mice. After 30 min of treating with Piperine orally, the Evans blue were intravenously injected, followed by the subcutaneous injection of C48/80 for 15 min, hindpaw edema and Evans blue extravasation were detected. After the hindpaw injection, Piperine application dose-dependently suppress partial cutaneous flare. The representative images of Evans blue extravasation demonstrated a weakened cutaneous flare in response to Piperine (Figure 2a). In response to 0, 2, 10, and 20 mg/kg of Piperine, hindpaw thickness increased by $39.07\% \pm 0.0999$, $28.73\% \pm 0.0632$, $28.18\% \pm 0.0554$, and $18.42\% \pm 0.0801$ (Figure 2b) and the relative Evans blue extravasation was 4.17 ± 1.20 , 3.42 ± 1.49 , 2.79 ± 0.77 , and 2.39 ± 0.31 (Figure 2c), respectively. Therefore, Piperine inhibited mice cutaneous flare by a dose-dependent manner.

3.2 | Piperine attenuated C48/80-induced body temperature hypothermia

The mice body temperature dropped rapidly and was slow to recover when anaphylaxis happened. Under the treatment of Piperine, the drop of mice body temperature were more modest and were proceeded by a quick recovery (Figure 3), which further explained that Piperine inhibited C48/80-induced hypersensitivity reactions.

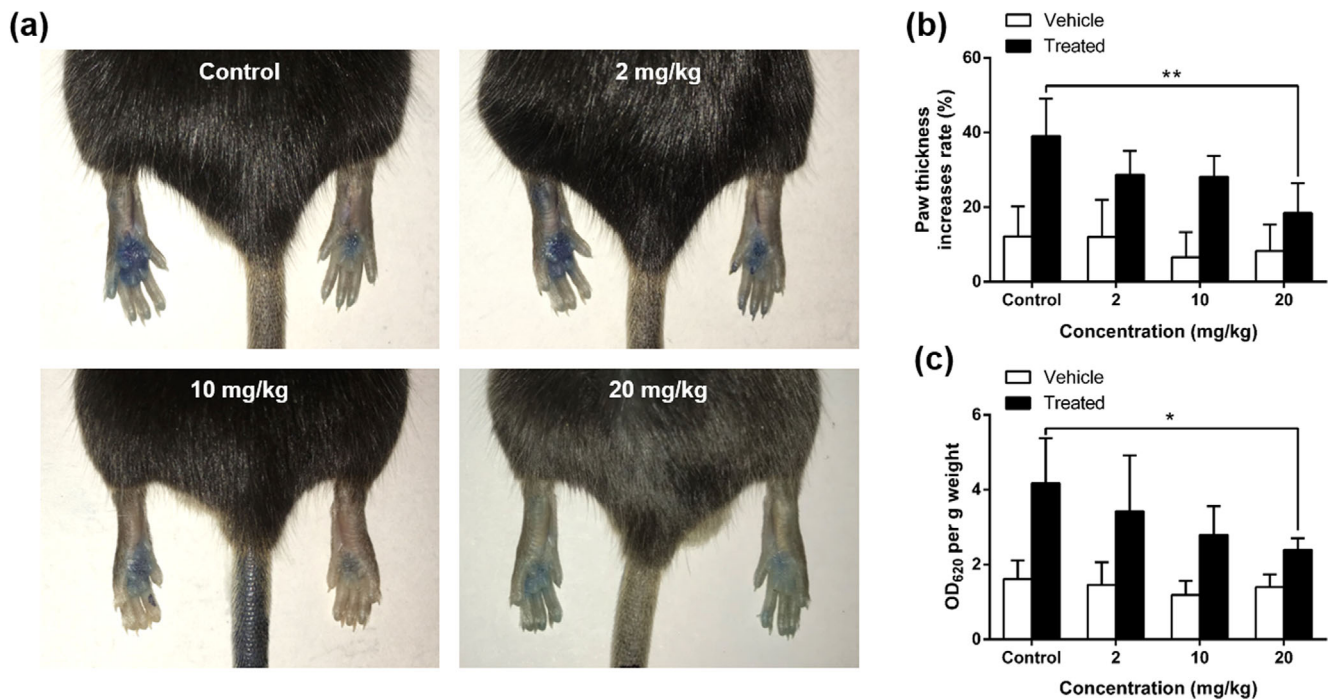


FIGURE 2 Handpaw thickness increased effect triggered by 30- μ M compound 48/80 (48/80) under the treatment of 30 min of Piperine orally in mice. (a) Representative images of Evans blue extravasation 15 min after the intraplantar injection of C48/80 in the left hindpaw or saline in the right hindpaw after 30 min of Piperine treatment orally with 0, 2, 10, or 20 mg/kg Piperine. (b) Quantification of increase in hindpaw thickness after 15 min. (c) Quantification of Evans blue extravasation into the hindpaw after 15 min. Statistical significance was defined at $p < .05$. (Data are presented as the means $\pm$ standard deviation. $n = 5$). OD, optical density

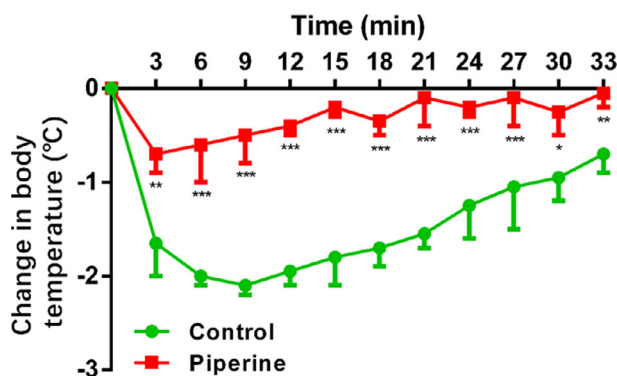


FIGURE 3 Changes in body temperature after intravenous injection of 10- μ M compound 48/80 under the treatment of 20 mg/kg Piperine orally. (Data are presented as mean $\pm$ standard error of the mean. $n = 4$)

3.3 | Piperine display little cytotoxic effect to mast cells

Based on the results of the cell viability assay by the Annexin V-FITC/PI Apoptosis Detection Kit, LAD2 cells were treated with 0, 12.5, 25, 50, 100, 200, or 400 μ M Piperine for 24 hr, and the cell viability was not affected significantly even at 400 μ M (Figure. 4).

3.4 | Piperine attenuated C48/80-induced Ca^{2+} mobilization in MPMCs

MPMCs are mature tryptase and chymase-expressing mast cells. The change of calcium ion concentration in the cell cytoplasmic can be effectively detected by the calcium label, which may determine the activation degree of the mast cells. MCPMs were treated with different concentration of Piperine orally (0, 50, 100, or 200 μ M) and incubated with Fluo-3 AM for 30 min. The cells were imaged before and after 30 μ M C48/80 stimulation. The average fluorescent intensity of a few mast cells was analyzed for each concentration (Figure 5a), meanwhile, the fluorescence intensity change value was measured by the maximum fluorescence intensity minus the minimum fluorescence intensity, which reflected a significant difference after treating with 50, 100, or 200 μ M Piperine (Figure 5b). The results showed that Piperine induced a dose-dependent decrease trend in C48/80-triggered Ca^{2+} mobilization in MPMCs.

3.5 | Piperine inhibited C48/80-induced degranulation in LAD2 cells

When mast cells were stimulated by the outside world, they degranulated particulate matter, which contained histamine, serotonin, cytokines, chemokines, and so forth. The preformed granule

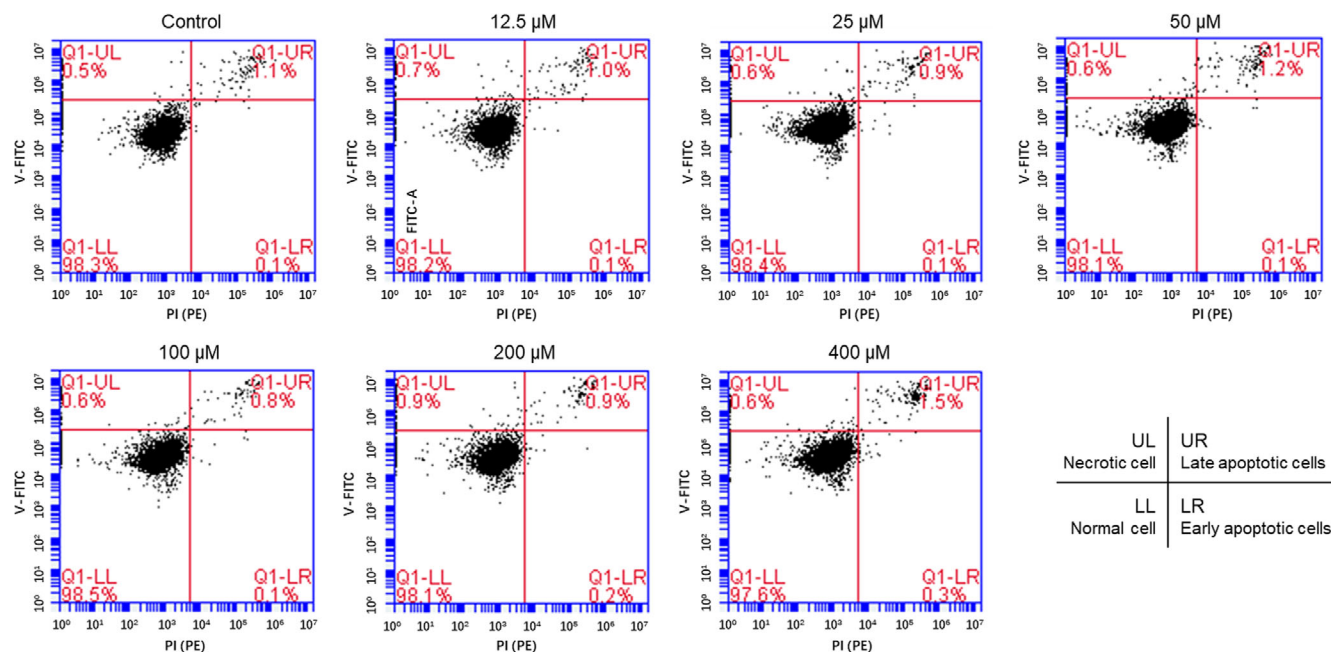


FIGURE 4 Viability of laboratory of allergic disease 2 cells treated by Piperine. The cells were incubated with increasing concentrations of Piperine (0–400 μ M) for 24 hr. The cell viability was detected by a flow cytometer. The results showed no cytotoxic effects on cell viability under the treatment of Piperine. LL, lower left; LR, lower right; PE, phosphatidylethanolamine; PI, propidium iodide; UL, upper left; UR, upper right

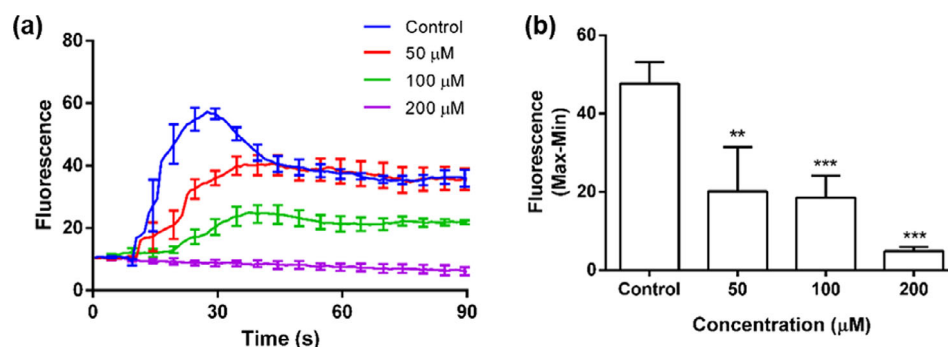


FIGURE 5 Calcium imaging in mouse peritoneal mast cells (MPMCs) triggered by C48/80 after the treatment of Piperine. (a) Representative Ca^{2+} imaging traces in MPMCs in response to 0, 50, 100 or 200 μ M Piperine and 30- μ g/mL compound 48/80. (b) Quantitative of changes in mast cells fluorescence intensity of (a). (Data are presented as mean $\pm$ standard error of the mean. $n = 3$ per concentration; 50 cells were counted per condition)

substances are immediately released and cause anaphylaxis reactions. The results showed that Piperine dose-dependently inhibited the release of β -hexosaminidase, histamine, PGD₂, TNF- α , IL-31, IL-8, and MCP-1 in C48/80-triggered LAD2 cells (Figure 6), indicating that Piperine can attenuate C48/80-induced mast cell degranulation.

3.6 | Piperine attenuated LAD2 cell degranulation induced by the endogenous and exogenous substances

Some small-molecule drugs, such as Ciprofloxacin, and endogenous peptidergic drugs, such as LL-37, can induce anaphylaxis reactions via

MRGPRX2. Hence, Ciprofloxacin and LL-37 triggered mast cell degranulation were detected under the treatment of Piperine. The results showed a significant reduction for β -hexosaminidase and histamine. Under the treatment of 0, 50, 100, or 200 μ M Piperine, the secretion of β -hexosaminidase were $37.10\% \pm 0.0083$, 36.68 ± 0.0090 , $29.65\% \pm 0.0181$, $15.35\% \pm 0.0068$, respectively, triggered by LL-37 (Figure 7a), and $23.32\% \pm 0.0137$, $18.30\% \pm 0.0055$, $16.78\% \pm 0.0025$, $12.80\% \pm 0.0151$, respectively, triggered by Ciprofloxacin (Figure 7b), respectively. Under the same conditions, the secretion of Histamine were 361.83 ± 40.98 , 248.10 ± 14.16 , 223.86 ± 16.99 , 173.08 ± 16.00 ng/mL, triggered by LL-37 (Figure 7c), and 174.32 ± 12.70 , 172.90 ± 5.30 , 147.06 ± 17.97 , 122.74 ± 15.71 ng/mL, triggered by Ciprofloxacin (Figure 7d),

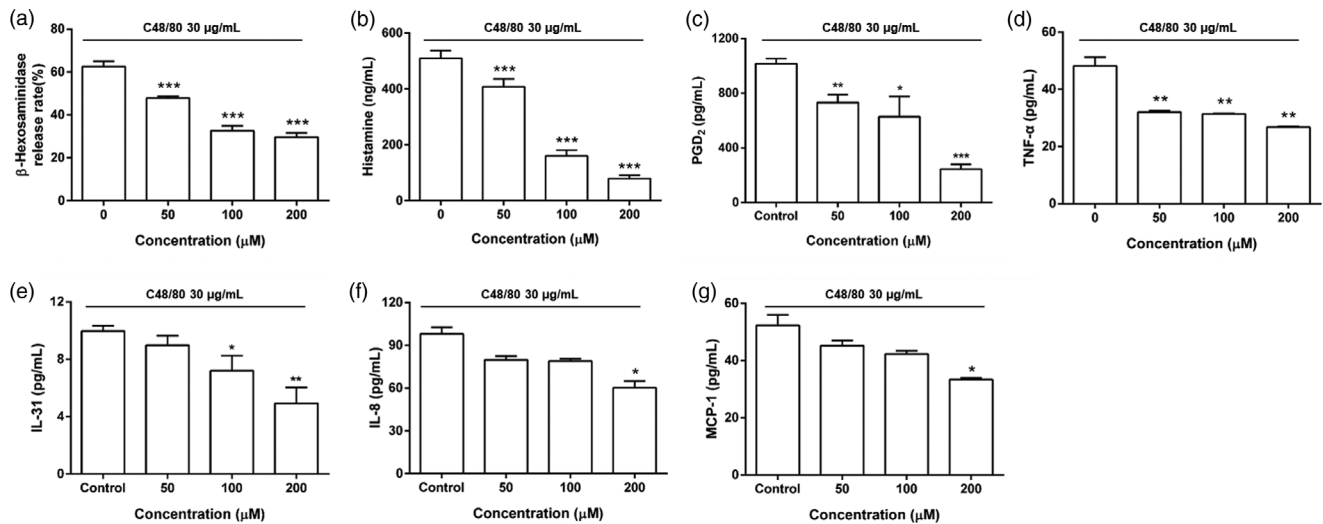


FIGURE 6 C48/80 induced mast cells degranulation under the treatment of Piperine. (a, b, c) Release of β -hexosaminidase (a), histamine (b) and PGD₂ (c) from LAD2 cells treated with 0, 50, 100 or 200 Piperine and 30 μ g/mL C48/80 for 30 min. (d, e, f, g) Release of TNF- α (d), IL-31 (e), IL-8 (f), and MCP-1 (g) from laboratory of allergic disease 2 cells treated with 0, 50, 100, or 200 μ M Piperine and 30- μ g/mL C48/80 for 6 hr. (Data are presented as the means $\pm$ standard deviation. $n = 5$). C48/80, compound 48/80; IL, interleukin; MCP, monocyte chemotactic protein; PGD₂, prostaglandin D₂; TNF- α , tumor necrosis factor- α

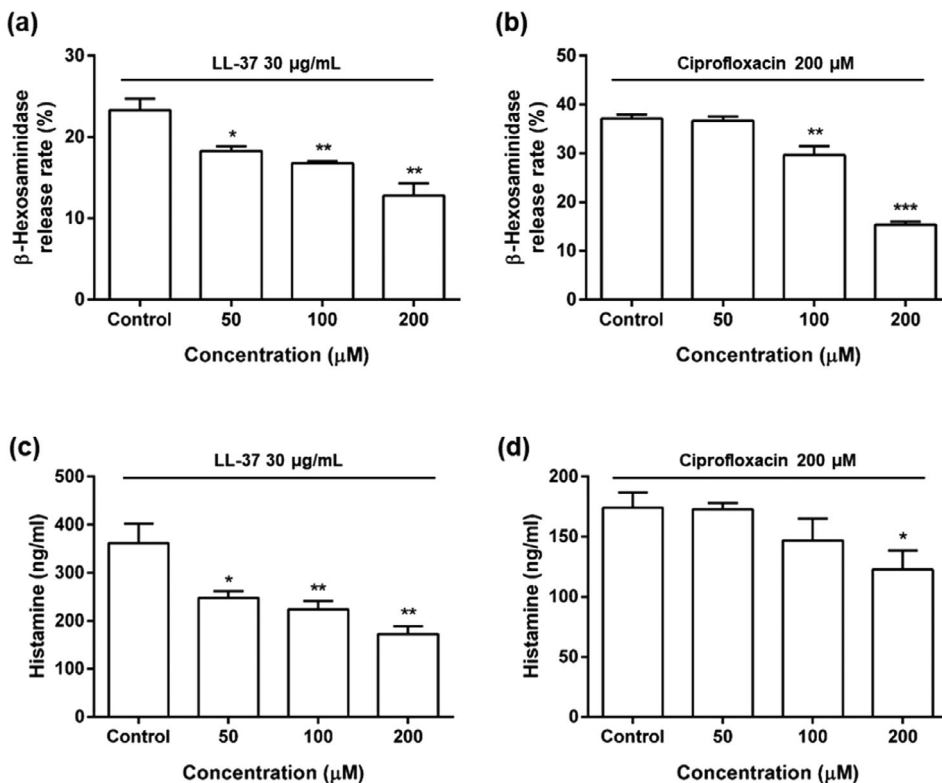


FIGURE 7 LL-37 or Ciprofloxacin induced mast cells degranulation under the treatment of Piperine. (a) β -hexosaminidase release of laboratory of allergic disease 2 (LAD2) cells treated with 0, 50, 100 or 200- μ M Piperine and 30 μ g/mL LL-37 for 30 min. (b) β -hexosaminidase release of LAD2 cells treated with 0, 50, 100 or 200 μ M Piperine and 200 μ M Ciprofloxacin for 30 min. (c) Histamine release of LAD2 cells treated with 0, 50, 100 or 200 μ M Piperine and 30- μ g/mL LL-37 for 30 min. (d) Histamine release of LAD2 cells treated with 0, 50, 100 or 200 μ M Piperine and 200 μ M Ciprofloxacin for 30 min. (Data are presented as the means $\pm$ standard deviation. $n = 5$)

respectively. Therefore, the secretion of β -hexosaminidase and histamine from LAD2 cells triggered by the endogenous and exogenous substances decreased dose-dependently in response to Piperine treatment, indicating that Piperine inhibited the release of inflammatory mediators from mast cells.

3.7 | Piperine regulating the activation of calcium fluctuations related pathway in mast cells

The mast cell intracellular calcium responses will activate the phosphoinositide 3-kinases pathway and the enzyme PLC γ 1

FIGURE 8 Effect of Piperine and C48/80 on the expression of PLC γ 1, IP3R, PKC, Akt, ERK, and P38 in laboratory of allergic disease 2 (LAD2) cells. (a, b) Western blot analysis of the expression levels of PLC γ 1, P-PLC γ 1, IP3R, P-IP3R, PKC and P-PKC (a), Akt, P-Akt, ERK, P-ERK, P38 and P-P38 (b) in LAD2 cells treated with 0, 50 or 100 μ M Piperine and 30- μ g/mL compound 48/80 for 30 min. (c) Quantification of (a, b) by densitometric analysis. (Data are presented as the means $\pm$ standard deviation. $n = 3$). Akt, protein kinase B; IP3R, inositol 1,4,5-triphosphate receptor; P-, antiphosphorylated-; PLC γ 1, phospholipase C γ 1; PKC, protein kinase C

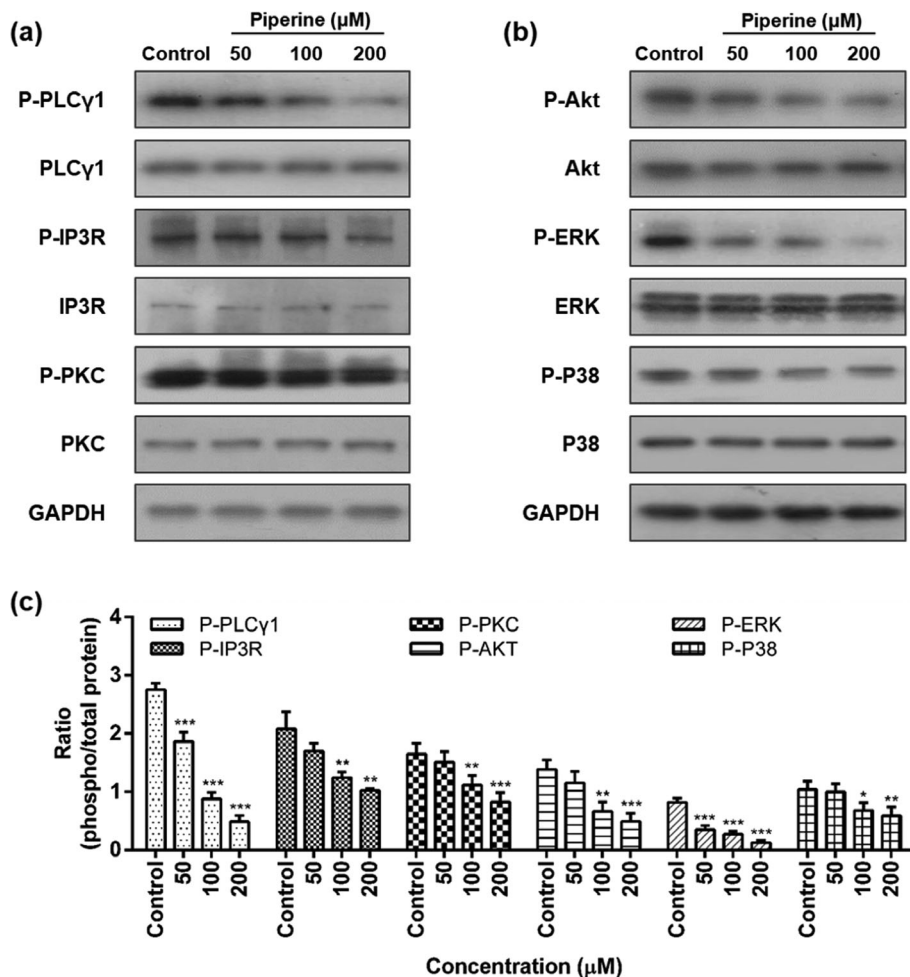
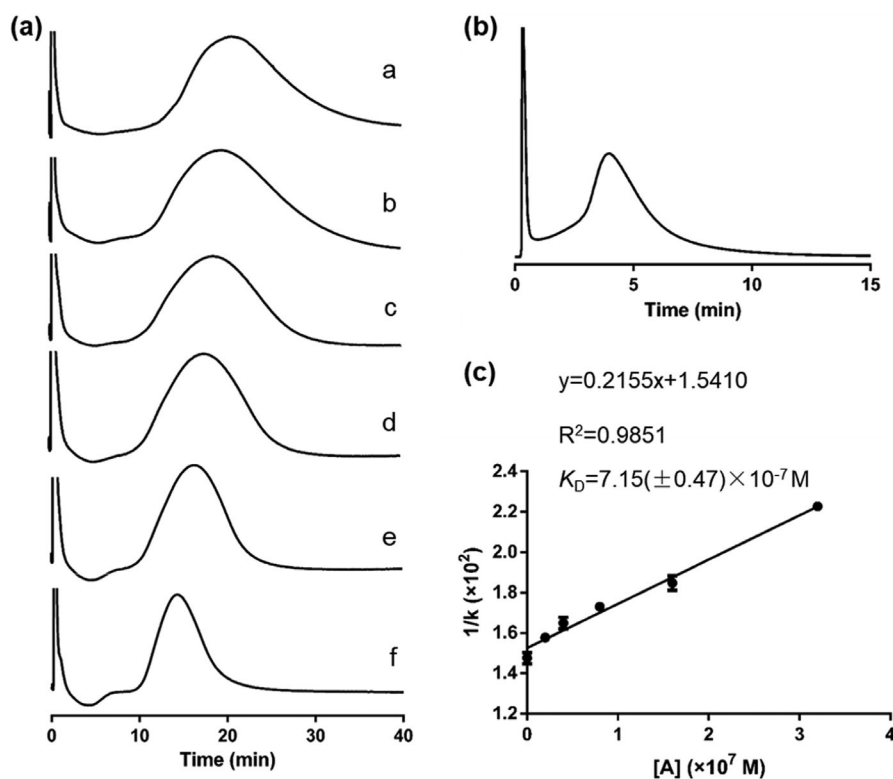


FIGURE 9 The K_D value of Piperine binding to MAS-related G protein-coupled receptor X2 (MRGPRX2). (a, b, c) K_D value calculated by carboxymethylcellulose method. (a) The MRGPRX2 cell membrane chromatogram of 500 μ M sinomenine hydrochloride using 0 (a), 2×10^{-8} (b), 4×10^{-8} (c), 8×10^{-8} (d), 1.6×10^{-7} (e), 3.2×10^{-7} M (f) of Piperine as mobile phase. (b) The MRGPRX2 cell membrane chromatogram of 500 μ M Piperine using water as mobile phase. (c) The regression curves achieved by plotting $1/k$ versus $[A]$, and the value was $7.15 (\pm 0.47) \times 10^{-7}$ M. (Data are presented as the means $\pm$ standard deviation. $n = 3$)



(Kalesnikoff & Galli, 2008), which could rise the mast cell intracellular calcium ($[Ca^{2+}]_i$). And promoting 1,4,5-trisphosphate binding to IP3R in the endoplasmic reticulum membrane causes the release of Ca^{2+} from endoplasmic reticulum stores, resulting in a decrease in the concentration of free Ca^{2+} in the ER. Meanwhile, the Akt, ERK, and P38 mitogen-activated protein kinase pathways were also activated during mast cell degranulation. Hence, the levels of phosphorylated PLC γ 1, IP3R, PKC, Akt, P38, and ERK were analyzed by western blotting. Under the treatment of 50, 100, or 200 μ M Piperine, the phosphorylation levels of these proteins were significantly downregulated in a dose-dependent manner when simultaneously triggered with 30 μ g/mL of C48/80 for 30 min, compared with which were treated with C48/80 alone (Figure 8). These results indicated that Piperine inhibited the PLC γ -IP3R related intracellular calcium responses.

3.8 | The K_D value of Piperine binding to MRGPRX2

CMC is an affinity chromatography method, which can be used for calculating K_D value of drug-receptor interaction. Here, we detected the Piperine-MRGPRX2 affinity using the CMC relative standard method. Gradient concentration of Piperine from 0 to 3.2×10^{-7} M were used for the mobile phase buffer, when the receptor of the CMC was saturated with different concentration of Piperine, the known agonist for MRGPRX2, sinomenine hydrochloride, was applied to the column, and the chromatographic retention peaks at corresponding concentrations can be obtained (Figure 9a). Then, we got the retention time (t_R) for sinomenine hydrochloride under the corresponding concentrations of Piperine. And Piperine has been proved that it can reserve in MRGPRX2/CMC, the retention time was 3.90 min (Figure 9b). According to Equation (1), the K_D value of Piperine binding to MRGPRX2 was $7.15(\pm 0.47) \times 10^{-7}$ M (Figure 9c).

4 | DISCUSSION

Mast cells were located at host-environment interfaces generally, and were divided into two types, mast cells containing the protease tryptase, which were predominantly found at mucosal sites, such as the lung and gastrointestinal tract, and mast cells containing the proteases tryptase and chymase, which were the predominant mast cell type of the skin (Bulfone-Paus et al., 2017). MRGPRX2 is expressed in mast cells containing the proteases tryptase and chymase and reacts to basic peptides, such as neuropeptides, host defense peptides, and certain drugs, by inducing rapid degranulation. MRGPRX2 triggering deliver calcium mobilization, as well as late responses, such as lipid mediator or chemokine/cytokine release (Subramanian, Gupta, & Ali, 2016b). Activation of mast cells via MRGPRX2 results in Ca^{2+} mobilization and the release of preformed and newly synthesized mediators that lead to anaphylactoid reactions, which may manifest ranging from milder skin reactions (e.g., maculopapular exanthema and urticaria) to severe systemic reactions

(e.g., bronchospasm, anaphylaxis, and shock; Chen et al., 2018; Subramanian et al., 2016a).

Piperine significantly reduced C48/80-triggered mice hindpaw cutaneous flare and body temperature hypothermia. There are few reports about the antianaphylactoid effect of Piperine. In 2014, Huang, et al. had proved that Piperine can inhibit antigen-induced rat basophilic leukemia cells degranulation (Huang et al., 2014). In 2015, Aswar et al. reported that Piperine prevented allergic responses by mast cell stabilization thereby inhibiting the release of mediators like histamine, IL-6, IL-1b, and immunoglobulin E, and has anti-allergic effect on ovalbumin-induced allergic rhinitis in mice (Aswar, Shintre, Chepurwar, & Aswar, 2015). Furthermore, in 2018, Preeti, et al. have reported that Piperine combined with Curcumin can reduce airway inflammation and against allergic asthma (Chauhan, Jaiswal, & Singh, 2018). However, all these reports told about that Piperine participated in mediating or suppressing type I allergic reactions in mice and rat basophilic leukemia cells. In this experiment, we reported that Piperine suppressed C48/80 induced human LAD2 cells activation and reduced C48/80-triggered mice hindpaw cutaneous flare and body temperature hypothermia, showing the antianaphylactoid effect of it in vitro and in vivo.

MRGPRX2 has been reported to induce calcium release from internal stores of IP3R production via PLC (Solinski et al., 2014). The agonist for MRGPRX2 activate pertussis toxin-dependent $G_{\alpha i}$ and pertussis toxin-independent $G_{\alpha q}$ pathways for MC responses (Subramanian et al., 2016b). Piperine can inhibit C48/80-triggered LAD2 cells calcium mobilization and the phosphorylation of IP3R, PLC, and PKC, thus suppressing mast cell degranulation and chemotaxis. Activation of MRGPRX2 also results in $G_{\alpha i}$ -dependent production of the pruritogenic cytokine IL-31 via the mitogen-activated protein kinase and Akt-dependent pathways (Subramanian et al., 2016b). In the present study, Piperine reduced the phosphorylation level of Akt, ERK and P38, and the release of IL-31. Therefore, it can be speculated that Piperine may has a certain inhibitory effect on pruritus. The antidegranulation effect of Piperine were also applicable to other drugs that cause anaphylactoid reactions, such as Ciprofloxacin and LL-37, indicated by a suppression of β -hexosaminidase and histamine release. K_D value is the basic parameter to evaluate the binding property of the drug-receptor (Alves et al., 2012). Gradient concentrations of Piperine can impact the retention time of MRGPRX2 specific agonist in MRGPRX2/CMC, which revealed the competitive binding effect of these two compounds.

Studies showed that cationic drugs had good affinity with MRGPRX2 (Solinski et al., 2014), which may be a good direction for finding its antagonists. From the structural formula of Piperine (Figure 1), we know that the tertiary amine makes it have cationic properties. Meanwhile, CMC method confirmed the affinity of Piperine to MRGPRX2, and Piperine can bind to MRGPRX2 competitively with its known agonist. Pharmacological and molecular biology experiments in this study have also confirmed its antianaphylactoid biological activity. Hence, we suspect that Piperine may be a MRGPRX2 antagonist, which could prebind to MRGPRX2 and inhibit the stimulation by other agonists. Thus, the activation of related downstream

signal pathways also could be prevented. The stabilization of mast cells could avoid drug-induced anaphylactoid reactions, such as pruritus, urticaria, and angioedema, and minimize the potential medicine risk.

In conclusion, Piperine exerts an inhibitory effect on drug-induced anaphylactoid reactions, which has potential to be a novel therapeutic agent for the treatment of drug-induced anaphylactoid reactions.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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