Polymyxin B and polymyxin E induce anaphylactoid response through mediation of Mas-related G protein–coupled receptor X2

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ABSTRACT

Polymyxin B (PMB) and polymyxin E (PME) are cyclic, peptide antibiotics which derived from various species of *Paenibacillus* (*Bacillus*) *polymyxa*. They are decapeptide antibiotics with an antimicrobial spectrum that includes Gram-negative bacteria, and reused as therapeutic agents due to the emergence of multidrug-resistant (MDR) Gram-positive bacteria. PMB or PME-induced anaphylactoid reactions in the clinic have been documented. However, the mechanism underlying anaphylactoid reaction induced by polymyxin has not yet been reported. Here, we report that human Mas-related G protein-coupled receptor X2 (MRGPRX2) and its mouse homologue Mas-related G protein-coupled receptor B2 (MrgprB2) are the receptors mediating the anaphylactoid response provoked by PMB and PME. We firstly investigated the anaphylactoid reactions induced by PMB and PME in LAD2 cells *in vitro* and *in vivo*, and found that treatment with PMB and PME led to significant release of mast cell granules such as histamine and β-hexosaminidase, secretion of pro-inflammatory cytokines, such as TNF-α and PGD2, and provocation of calcium flux in LAD2 cells. Furthermore, treatment with PMB and PME led to reduced release of β-hexosaminidase in MRGPRX2 knockdown-LAD2 cells, and obvious increased calcium release in MRGPRX2 overexpressing HEK293 cells, which suggested that MRGPRX2 are involved in mast cell activation provoked by PMB or PME. *In vivo*, MRGPRB2 knockout mice exhibited lower pseudo-allergic reactions than wild type mice. Activation of MrgprB2 also triggers increased capillary permeability and paw swelling. Our results elucidated the role of MRGPRX2 in PMB and PME-induced anaphylactoid response and suggested that MRGPRX2 as a potential therapeutic target to control the anaphylactoid reactions which triggered by PMB or PME.

1. Introduction

Drug allergy is one type of adverse reaction to drugs with several clinical presentations. Among all reported allergy cases, some cases are pseudo-allergic (anaphylactoid) reactions [1,2]. Like anaphylactic response, anaphylactoid response is an off-target effect of drugs. Anaphylactoid reactions are different from anaphylactic response that may occur after the first exposure to the agents by nonantigen/antibody-mediated mechanisms. Its occurrence is often related to high dose drug administration or rapid injection. In other words, the extent of the reactions is usually dose-dependent [1].

As bone marrow-derived immune effector cells, mast cells are found abundantly in mucosal members, skin and tissues which interface with the external environment [3,4]. They are the primary effectors involved in anaphylactoid reactions. In certain situations, many clinical drugs can elicit anaphylactoid responses and result in severe adverse drug reactions. Following the reactions, direct release of mast cell mediators

and activation of the inflammatory cascades will be detected. The occurrence of this effect do not depend on the cross-talk with surface IgE antibodies on mast cells [5–8].

Activated mast cells lead to mast cell granules, which components include β-hexosaminidase, histamine, and several other inflammatory factors such as proteoglycans, eicosanoids, proteases and varieties of chemotactic and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), prostaglandin D2 (PGD2), interleukin (IL)8, IL-6, IL-4 [9–11]. Among them, histamine is a major mediator which can trigger inflammatory biological response including increasing vascular permeability, vasodilation, tissue edema, contraction of bronchial, and increased mucus production [12,13].

Polymyxins are a group of polycationic antimicrobial lipopeptide biosynthesized by bacteria belonging to the genus *Bacillus* that include polymyxin A, B, C, D, E. Among them, polymyxin B (PMB) and polymyxin E (PME, also known as colistin methanesulfonate clinically), are the major representatives of polymyxins that have been used in clinical

practice since 1950s [14]. Subsequently, the emergence of significant adverse effects such as neurotoxicity, nephrotoxicity, urticaria rash limited their uses [15]. However, the increase of infections caused by global multidrug-resistant organisms has led to renewed use of polymyxins in the clinic. Polymyxins exhibit highly bactericidal activities and are currently the last-resort antibiotics for treating infections caused by multidrug-resistant Gram-negative bacteria including *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [16–18]. The cases of anaphylactic and anaphylactoid reactions caused by PMB treatment, such as mild itching, episodes of rash, contact dermatitis (eczema and erythematous eruption) following, have been reported previously [19–22]. In addition, polymyxins have been reported as mast cell activators, which can stimulate a release of histamine and other mediators in rats or dogs to mediate several inflammatory or allergic response [23,24]. However, the mechanism underlying anaphylactoid reaction induced by polymyxins was not completely understood. To address this problem, we examined the anaphylactoid reactions induced by PMB and PME, and characterized the effects of MRGPRX2 involved in this process.

2. Materials and methods

2.1. Cells culture and animals

Human mast LAD2 (Laboratory of Allergic Diseases 2) cells, gifted by A. Kirshenbaum and D. Metcalfe from NIH (MD, USA) were maintained in StemPro-34 medium (Life Technologies, Marietta, OH) supplemented with StemPro-34 supplement, 100 ng/mL recombinant human stem-cell factor (SCF, Sino Biological, Beijing, P.R. China), 2 mM L-glutamine, 50 mg/mL streptomycin and 100 U/mL penicillin at 37 °C in a 5% CO_2 incubator (Thermo Electron Corp., Marietta, OH).

C57BL/6 mice as well as genetically MrgprB2-deficient mice (MrgprB2^{MUT}) were kindly provided by Dr. X. Dong (Johns Hopkins University, USA). All animals were housed in an aseptic condition and administrated with sterilized water and food. All animal experiments were conducted according to the guidelines and approvals of the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

2.2. Drugs

PMB sulfate, PME (Colistin sulfate salt), Compound 48/80 and Evans blue were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO, USA), Human PGD2 ELISA Kit (No. 512031) was obtained from Cayman chemical Co (Ann Arbor, Michigan, USA) and Human TNF-α ELISA Kit was purchased from ExCell Biology, Inc. (Shanghai, P.R. China). Fura-2 and Fluo-4 AM were purchased from Molecular Probes (Thermo Fisher Scientific Co., Carlsbad, USA).

2.3. LAD2 degranulation assay

LAD2 cells were harvested, resuspended in HEPES buffer (10 mM HEPES, 137 mM NaCl, 0.38 mM $Na_2HPO_4.7H_2O$, 2.7 mM KCl, 1.3 mM $MgSO_4.7H_2O$, 1.8 mM $CaCl_2.H_2O$, 5.6 mM glucose, 0.4% BSA, pH 7.4), and then seeded into 96-well round bottom plate at a density of 2.5×10^4 cells/well, PMB and PME at indicated concentrations were added to stimulate cells for 30 min at 37 °C. The 96-well plate was centrifuged, 50 μL of supernatants of each well were transferred to another plate. The cells pellets were lysed with 50 μL of Triton X-100, and 50 μL of lysate were transferred to another plate. Supernatant and lysate were then incubated with equivalent volume of *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide (PNAG, Sigma-Aldrich) substrate solution for 90 min. At the end of incubation, the reaction was terminated with 0.4 M glycine (pH 10.7), and absorbance was read at 405 nm with a microplate spectrophotometer. The percentage of β-hexosaminidase released into cell supernatant after stimulation of drugs was calculated as a percent of total content.

2.4. Determination of histamine release

Histamine released from LAD2 cells or in peripheral blood was determined by using LC-MS/MS method developed by Han et al. previously [25]. LAD2 cells were seeded in 6 well plate at a density of 1×10^5 cells/well, drugs at different concentrations were added to cells. After 60 min incubation, 96-well plate was centrifuged at 1000 rpm for 5 min, and 50 μL of cell medium was collected and subjected to LC-MS/MS to investigate the content of histamine released from LAD2 cells after treatment with tested drugs. Before LC-MS/MS, the samples (50 μL of cell medium) were premixed with 2-fold volume of acetonitrile containing histamine-d4 and then centrifuged at 10000 rpm, 4 °C for 10 min, then the supernatant was collected to investigate histamine.

Histamine released in peripheral blood after stimulation of mice with drugs were also determined in this study. Mice were administrated with drugs at indicated concentrations, 30 min later, the peripheral blood was collected, and centrifuged at 12000 rpm, 4 °C for 5 min. Serum was collected for LC-MS/MS analysis.

2.5. ELISA assay

TNF-α and PGD2 secreted by LAD2 cells were detected with ELISA kits. LAD2 cells were seeded in 96-well plate at a density of 2.5×10^4 cells/well, and treated with PMB and PME respectively at indicated concentrations. Incubated for 48 h at 37 °C in a 5% CO_2 incubator, cell supernatant was collected and subjected to ELISA assay according to instructions.

2.6. EC50 determination

LAD2, MRGPRX2/HEK293 and MrgprB2/HEK293 cells were seeded in black 96-well plate. Cell medium was removed after overnight incubation and cells were incubated in FLIPR Calcium 5 solution (Molecular Devices), which was diluted in Hank's balanced salt solution (HBSS) with 20 mM HEPES, pH 7.4, at 37 °C for 1 h, 1 h later, cells were placed at room temperature for 15 min before measuring in Flexstation 3 (Molecular Devices, San Jose, CA, USA). The fluorescence of each well was detected for 120 s, the tested drugs diluted in HBSS plus HEPES were added to the wells 30 s after detection began. EC50 values were determined by normalizing to the peak fluorescent response to the drugs in one experiment.

For LAD2 cells, cell density were 1×10^5 cells/well, the final concentrations of PMB and PME were 8.6, 17.3, 34.6, 69.2, 138.5, 277.0, 1108.0 μg/mL, and 2.2, 8.7, 34.6, 138.5, 277.0, 554.0, 2216.0, 4432.0 μg/mL, respectively. For MRGPRX2/HEK293 cells, cells were seeded at a density of 5×10^4 cells/well, the final concentrations of PMB and PME were 1.1, 2.2, 4.3, 8.7, 17.3, 34.6, 69.2, 138.5 μg/mL, and 0.008, 0.03, 0.1, 0.5, 2.2, 8.7, 34.6, 69.2 μg/mL, respectively. For MrgprB2/HEK293 cells, cells were seeded at a density of 5×10^4 cells/well, the final concentrations of PMB and PME were 34.6, 69.2, 277.0, 554.0, 1108.0, 2216.0, 4432.0, 8864.0 μg/mL, and 17.3, 277.0, 554.0, 1108.0, 2216.0, 8864.0, 17728.0 μg/mL, respectively.

2.7. siRNA interference of MRGPRX2 in LAD2 cells

For siRNA experiment, LAD2 cells were transiently transfected with siRNA against MRGPRX2 and control siRNA using Lipofectamine 2000 (Life Technologies, Marietta, OH) according to the manufacturer's instruction. After 48 h transfection, MRGPRX2 expression was confirmed by qPCR and western blotting, then the cells were used for β-hexosaminidase release assay.

2.8. Calcium imaging

LAD2, MRGPRX2 expressing HEK293 (MRGPRX2/HEK293) cells and MrgprB2 expressing HEK293 (MrgprB2/HEK293) cells were seeded in glass coverslips coated with 0.1 mg/mL Poly-D-Lysine at a density of 1×10^4 cells/well, after 24 h of incubation, culture medium was removed, calcium imaging buffer (CIB: NaCl 125 mM, KCl 3 mM, CaCl$_2$ 2.5 mM, MgCl$_2$ 0.6 mM, HEPES 10 mM, glucose 20 mM, NaHCO$_3$ 1.2 mM, sucrose 20 mM, brought to pH 7.4 with NaOH) was added to the coverslips, and cells were loaded with a fluorescent Ca^{2+} indicator, Fura-2 AM or Fluo-4 AM (Molecular Probes, Carlsbad, USA), along with 0.02% Pluronic F-127, (Molecular Probes, Carlsbad, USA) for 45 min at 37 °C. The fluorescent Ca^{2+} indicator was changed to CIB before imaging. Cells loaded Fura-2 were observed during 340 and 380 nm excitation, and cells loaded Fluo-4 were observed at 488 nm excitation.

2.9. Evans blue extravasation assay

C57BL/6 mice up to 8 weeks (20–25 g) of age were used to determine the hindpaw swelling and Evans blue extravasation. Mice were anaesthetized by intraperitoneal injection 50 mg/kg phenobarbital. After 10–15 min, anaesthetized mice were intravenously injected 50 mL of 12.5 mg/mL Evans blue in saline. After 5 min, 5 μL of drugs at indicated concentrations were injected subcutaneously into the right hindpaw, equivalent saline as a control were injected into the left hindpaw with the same method. At the end of injection, the thickness of paws was measured using a vernier caliper to determine whether the paw is swelling. 15 min later, the thickness of paws was measured again. Then the mice were killed and then the paws were collected, dried in a drier at 60 °C for 24 h and weighed. The dried paws were cut into pieces and soaked into acetone/saline (7:3) overnight to extract the dye. The next day, the samples were centrifuged at 2000 rpm for 20 min, 100 μL of the supernatant were collected and OD value was measured at the wavelength of 620 nm with a microplate reader.

2.10. Statistical analysis

Quantitative data are shown as mean ± standard error of the mean (SEM). All data analyses were done with Prism software (GraphPad, La Jolla, CA, USA), and statistical comparisons were done with SPSS 19.0 (IBM, Chicago, IL, USA). The ANOVA was used for determining the statistical significance of data compared with the control group. $P < 0.05$ was taken as statistically significant.

3. Results

3.1. PMB or PME induced degranulation of LAD2 cells

Degranulation of mast cell, an essential process of drugs-triggered anaphylactoid reaction, was verified by determining the release of some factors involved in this process, such as β-hexosaminidase, histamine, TNF-α, PGD2. β-hexosaminidase released from LAD2 cells was investigated after treatment of LAD2 cells with PMB or PME for 30 min. The results showed that PMB or PME led to the release of β-hexosaminidase in a dose-dependent manner (Fig. 1a). Histamine released from LAD2 cells treated with PMB or PME for 60 min was determined by HPLC-MS/MS. The results showed that the concentration of histamine was elevated gradually along with the increase of concentration (Fig. 1b). ELISA assay was used to determine the release of TNF-α and PGD2, the concentration of TNF-α and PGD2 in LAD2 cells treated with 138.5 μg/mL of PMB or 554.0 μg/mL of PME were significantly raised compared with vehicle (Fig. 1c and d).

3.2. PMB and PME induces Ca^{2+} mobilization in LAD2 human mast cells

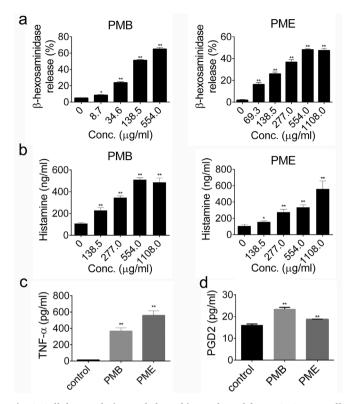Calcium mobilization is an important event in mast cell

Fig. 1. Cell degranulation and chemokines released from LAD2 mast cells activated by PMB and PME. LAD2 cells were treated with PMB or PME at indicated concentrations prior to determination of β-hexosaminidase, histamine, TNF-α and PGD2. Quantitative data are presented as mean ± SEM for triplicate experiments. *P < 0.05; **P < 0.01 compared with control.

deregulation. The changes in Ca^{2+} concentration in LAD2 cells were investigated through calcium imaging in LAD2 cells probing with Fluo-4 AM, significant elevated Ca^{2+} fluorescence and the number of activated cells were observed in LAD2 cells after treatment with PMB or PME. It indicated that PMB and PME induced Ca^{2+} mobilization in LAD2 cells (Fig. 2a and b).

To determine EC50 values of PMB and PME in activating LAD2 cells, calcium 5 assay was carried out in this study. From the curve shown in Fig. 2c and d, EC50 values of PMB and PME were 31.7 μg/mL and 31.8 μg/mL, respectively.

3.3. MRGPRX2 mediated anaphylactoid responses induced by PMB or PME

MRGPRX2 is over-expressed in LAD2 cells, which could be activated by PMB or PME, we suspected that PMB and PME activated LAD2 cells to release Ca^{2+} via MRGPRX2. To further confirm the role of MRGPRX2 in PMB or PME triggered anaphylactoid responses, siRNA transfection was used to knock down MRGPRX2 expression in LAD2 cells, and then β-hexosaminidase release from MRGPRX2-knockdown-LAD2 cells was determined. We observed that MRGPRX2 expression was greatly decreased in MRGPRX2 siRNA transfected cells compared with wild-type LAD2 cells (Fig. 3a). As expected, LAD2 degranulation assay showed that β-hexosaminidase release from LAD2 cells declined significantly along with the decrease of MRGPRX2 expression (Fig. 3b and c).

3.4. PMB and PME induces Ca^{2+} mobilization in MRGPRX2 overexpressing HEK293 cells

Calcium release was detected in MRGPRX2 overexpressing HEK293 cells, the results showed that Ca^{2+} concentration was increased obviously in cells treated with PMB or PME (Fig. 4a and b), whereas there were no changes in HEK293 cells (data not shown). Also,
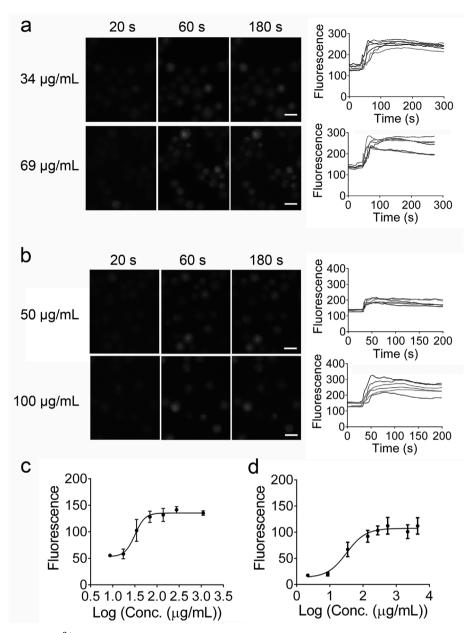
Fig. 2. The changes in intracellular Ca²⁺ concentration in LAD2 cells induced by PMB and PME. a and b, Left, Representative intracellular calcium images of LAD2 cells before and after simulation by PMB (a) and PME (b) at indicated concentrations. Scale bar, 10 μm. Right, representative imaging traces and the quantification of responding cells. Each color line represents an individual cell. c and d, The dose-response curve of PMB (c) and PME (d) associated with pseudo-allergic reactions to activate LAD2 cells. The EC50 values were determined from this assay. Data are expressed as mean ± SEM for triplicate experiments.
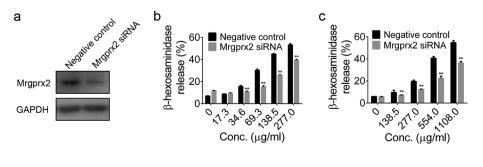

Fig. 3. MRGPRX2 mediated PMB and PME induced pseudo-allergic reactions. a, The blot of MRGPRX2 protein in LAD2 cells after MRGPRX2 knockdown by siRNA transfection. b, LAD2 cells were transfected with siRNA against MRGPRX2 followed by simulation of PMB at indicated concentration, β-hexosaminidase release was assessed by LAD2 degranulation assay. c, LAD2 cells were transfected with siRNA against MRGPRX2 followed by simulation of PME at indicated concentration, β-hexosaminidase release was assessed by LAD2 degranulation assay. Data are expressed as mean ± SEM for triplicate experiments. *P < 0.05; **P < 0.01 compared with control.
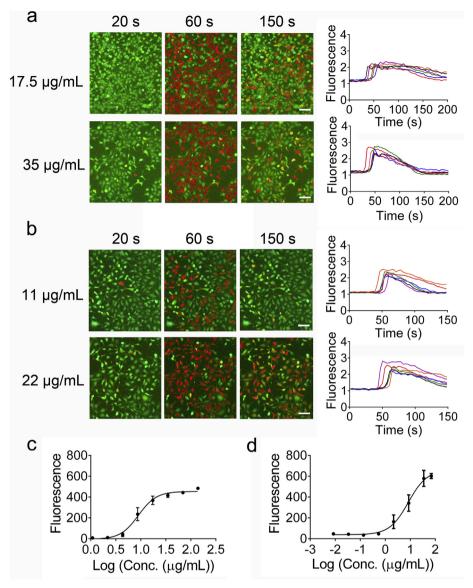
Fig. 4. The changes in intracellular Ca²⁺ concentration in MRGPRX2/HEK293 cells induced by PMB and PME. a and b, Left, Representative intracellular calcium images of MRGPRX2/HEK293 cells before and after simulation by PMB (a) and PME (b) at indicated concentrations. Scale bar, 50 μm. Right, representative imaging traces and the quantification of responding cells. Each color line represents an individual cell. c and d, The dose-response curve of PMB (c) and PME (d) to activate MRGPRX2/HEK293 cells. The EC50 values were determined from this assay. Data are expressed as mean ± SEM for triplicate experiments.

we measured the EC50 in MRGPRX2/HEK293 cells, from the curve, EC50 value in PMB and PME was 8.9 μg/mL and 12.2 μg/mL (Fig. 4c and d), which is much lower than that in LAD2.

3.5. PMB or PME induced histamine release in mice

We then explored the effect of drugs on histamine release *in vivo*. The concentration of histamine releasing into peripheral blood after 30 min of drugs injection was determined with HPLC-MS/MS. The results showed that PMB or PME exhibited an obvious induction on histamine release into peripheral blood of mice (Fig. 5a and e).

3.6. MrgprB2 mediated anaphylactoid responses induced by PMB or PME

A passive cutaneous anaphylaxis mouse model was performed to validate the effect of PMB or PME over again *in vivo*. The wild-type C57BL/6 mice and MrgprB2 knockout mice were injected intravenously Evans blue before injection subcutaneous of PMB or PME in the left paw, and saline in right paw as a control. Extensive extravasation and

paw swelling was observed in left paw of wild-type mice but not in MrgprB2 knockout mice after 15 min of injection subcutaneous of PMB or PME (Figure b and f). We then determined the dye leakage by measurement of OD value at a wavelength of 620 nm and extent of paw swelling by measurement of the thickness of paw before and after injection of drugs. The results showed that both OD value and paw thickness were significantly increased in wild-type mice, whereas there were almost no changes in MrgprB2 knockout mice (Figure c, d, g and h).

We next confirmed whether this effect was due to the activation of MrgprB2 in mast cells or not, the ability of PMB or PME induced Ca²⁺ release in MrgprB2 overexpressing HEK293 cells was investigated by calcium imaging and calcium 5 assay. The elevated fluorescence was observed in MrgprB2/HEK293 cells (Fig. 6a and b), and EC50 value in this cells was 1602 and 1091 μg/mL (Fig. 6c and d), which indicated PMB or PME could active MrgprB2/HEK293 cells.
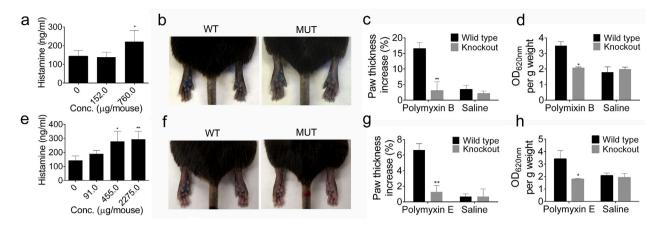
Fig. 5. MrgprB2 mediated PMB and PME-induced cutaneous pseudo-allergic reactions in mice. a, Histamine release from the peripheral blood of mouse induced by PMB at indicated concentrations. b, Representative images of Evans blue stained extravasation 15 min after intraplantar injection of PMB (left, 0.2 mg/mL) or saline (right). c, Quantification of Evans blue leakage into the paw 15 min after injection of PMB. d, Quantification of paw thickness increase 15 min after injection of PMB. e, Histamine release from the peripheral blood of mouse induced by PME at indicated concentrations. f, Representative images of Evans blue stained extravasation 15 min after intraplantar injection of PME (left, 1 mg/mL) or saline (right). c, Quantification of Evans blue leakage into the paw 15 min after injection of PME. d, Quantification of paw thickness increase 15 min after injection of PME. Data are expressed as mean ± SEM for triplicate experiments. *P < 0.05; **P < 0.01 compared with control or wild-type mice.

4. Discussion

Anaphylactoid reactions, a severe responses caused by direct action of substances (drugs, toxins), occur through a direct non-immune-mediated release of mediators, and they present with clinical symptoms similar to those of anaphylaxis. Antibiotics administered perioperatively can cause immunologic or non-immunologic generalized reactions. Zhang et al. [26] have reported that anaphylactoid reactions induced by some typical antimicrobials, such as sisomicin sulfate, terbinafine hydrochloride, sulfamethoxazole. This study is the first to evaluate the effect of PMB and PME on induction of anaphylactoid reactions *in vitro* and *in vivo*, and provides direct evidence that involvement of MRGPRX2 and MrgprB2 in this process.

PMB and PME are the two clinical use polypeptide antibiotics. It has been reported that polymyxins caused anaphylactoid reactions in patients with high frequency, it also caused the release of histamine from mast cells isolated from rats [24]. These data suggest that direct histamine release occurs with polymyxins administration, but not immunological. In this study, we assessed degranulation from the LAD2 human mast cells induced by PMB and PME, including β-hexosaminidase, histamine, TNF-α and PGD2. In accordance with the literature, we observed that degranulation of LAD2 cells occurred immediately after stimulating with PMB and PME by non-immunological mechanisms, which verified the phenomenon of anaphylactoid reaction induced by PMB and PME in vitro.

The mechanism of anaphylactoid reactions has been reported in some literature. Basic polycationic peptides are considered to stimulate βγ Subunit of spertussis toxin-sensitive G proteins (Gi2 and Gi3) on the mast cell surface via signal pathways involving phospholipase C-β, phospholipase C-γ, which then promote intracellular Ca^{2+} mobilization that leads to exocytosis of preformed granules (for example, histamine) and the release of arachidonate [27].
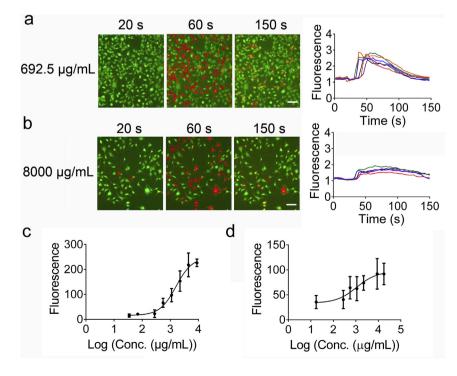

Fig. 6. The changes in intracellular Ca^{2+} concentration in MrgprB2/HEK293 cells induced by PMB and PME. a and b, Left, Representative intracellular calcium images of MRGPRX2/HEK293 cells before and after simulation by PMB (a) and PME (b) at indicated concentrations. Scale bar, 50 μm. Right, representative imaging traces and the quantification of responding cells. Each color line represents an individual cell. c and d, The dose-response curve of PMB (c) and PME (d) to activate MrgprB2/HEK293 cells. The EC50 values were determined from this assay. Data are expressed as mean ± SEM for triplicate experiments.

Recently, Mas-related G protein-coupled receptor X2 (MRGPRX2), a member of G protein-coupled receptors (GPCRs), is involved in most biological and pathophysiological processes and highly expressed in human mast cells. It has been proved to be a receptor to mediate activation of mast cells induced by many small-molecule drugs and peptidergic drugs. MRGPRX2 activation induces intracellular calcium release and degranulation in human mast cell line [28]. This provides a further understanding to know about the mechanism of anaphylactoid reactions induced by many drugs. In the current study, we first investigated the effect of PMB and PME on human mast cells LAD2 to elucidated their anaphylactoid reactions, and found that Ca^{2+} flux mobilization and granules release were observed in LAD2 cells after simulation of PMB and PME. LAD2 cell line is a MRGPRX2 over-expressing cell line and MRGPRX2 is proved to be involved in anaphylactoid reactions. Furthermore, transfection of LAD2 cells with siRNA encoding MRGPRX2 rendered them less responsive to PMB and PME for degranulation. For this reason, we assumed that MGRPRX2 might be a target to mediate PMB and PME's anaphylactoid reactions. To confirm this hypothesis and figure out its mechanism, MRGPRX2 overexpressing HEK293 cells were used to clarify whether PMB and PME activated intracellular calcium release via MRGPRX2. We found that PMB and PME exerted a strong induction of Ca^{2+} flux mobilization in MRGPRX2 overexpressing HEK293 cells, and IC50 value of PMB and PME was much lower than that in LAD2 cells, but this response was absent in non-transfected HEK293 cells. That was to say, PMB and PME activated degranulation and calcium release of human mast cell line mediated by MRGPRX2, subsequently triggered occurrence of anaphylactoid reactions. Based on above research, we authenticated MRGPRX2 was involved in anaphylactoid response triggered by PMB and PME.

Consistent with studies in vitro, we found that PMB and PME induced histamine release in mice. The mechanism for the mast cell activation is likely to be similar for both humans and mice. However, MRGPRX2 is not expressed in mouse mast cells. McNeil et al. [28] reported mouse protein-coupled receptor Mrgprb2 expressing in mouse connective tissue-type mast cells was identified the orthologue of the human MRGPRX2, which can mediate activation of mouse mast cell to elicit anaphylactoid responses such as histamine release, inflammation and airway contraction. MrgprB2 can be activated by a range of FDA-approved drugs known to induce allergic-type injection site reactions. Thus, it is reasonable to presume that PMB and PME-induced anaphylactoid reactions in mice might be mediated by mouse MrgprB2. Subsequently, investigations in wild-type and MrgprB2 mice by Evans blue assay confirmed that PMB and PME were able to elicit significant local cutaneous flare reactions in wild-type mice, and these reactions were almost lost in MrgprB2-knockout mice. Furthermore, PMB and PME exerted an induction of Ca^{2+} flux mobilization in MrgprB2 overexpressing HEK293 cells. These findings suggested that PMB and PME activate mouse mast cells via MrgprB2.

In this study, we have evaluated the roles of MRGPRX2 in PMB and PME-induced anaphylactoid response. Human MRGPRX2, which is expressed on mast cells, functionally resembles MrgprB2 on mouse mast cell. PMB and PME can activate MRGPRX2 in cell membrane, and the activation of MRGPRX2 subsequently trigger intracellular Ca^{2+} signaling, the increase in intracellular Ca^{2+} concentration followed by the degranulation of mast cells results in the occurrence of anaphylactoid responses. Therefore, it might be a promising way to prevent PMB and PME induced drug anaphylactoid reaction by targeting human MRGPRX2.

Declaration of interests

- The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
- The authors declare the following financial interests/personal

relationships which may be considered as potential competing interests.

Conflicts of interest

The authors declared no conflicts of interest.

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