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A mast-cell-specific receptor mediates Iopamidol induced immediate IgE-independent anaphylactoid reactions

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ABSTRACT

Iopamidol is a radiographic contrast media which caused a very high incidence of anaphylactic reactions. Mast cells are sentinel cells in host defense reactions during immediate hypersensitivity responses and anaphylactic responses. Mas-related G protein-coupled receptor X2 (MRGPRX2) is a kind of mast cell specific receptor, which triggers mast cell degranulation in anaphylactic reactions. Mice MrgprB2 is a homologous gene of MRGPRX2. We sought to better understand the anaphylactic reactions induced by Iopamidol and the mechanisms involving MRGPRX2. The MRGPRX2-related anaphylactic reactions induced by Iopamidol were investigated using the hindpaw swelling and extravasation assay *in vivo* and a calcium imaging assay was used for mast cell intracellular calcium responses detection and mast cell release of anaphylactic mediators, such as β-hexosaminidase, histamine and TNF-α, was also detected *in vitro*. The mast cell deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice and MrgprB2 knockout mice exhibited a reduced Iopamidol-induced inflammation effect compared with wild type mice. Furthermore, human mast cells that express MRGPRX2 were activated by Iopamidol in a dose-dependent manner, meanwhile MRGPRX2 knockdown mast cells showed reduced intracellular calcium responses and anaphylactic mediators release effect. It could be concluded that Iopamidol-induced anaphylactoid reactions were MRGPRX2 mediated to provoke mast cells Ca^{2+} mobilization and degranulation.

1. Introduction

Iodinated contrast media (ICM) is widely used in angiography and imaging diagnosis to improve imaging resolution between tissues. Globally, > 75 million diagnostic radiographic studies use ICM each year [1,2]. The incidence of hypersensitivity reactions has increased with the increasing use of ICM in different types of radiologic examinations [3,4]. Hypersensitivity reactions may be divided into non-immediate responses (> 1 h of administration) and immediate (< 1 h). Acute adverse reactions (AARs) to ICM occur within 1 h of injection and vary in range of intensity from mild to severe reactions, such as anaphylaxis, and the severe immediate reactions occur with a frequency of 0.02–0.04% [5]. ICM can generally be classified into ionic (high-osmolar) and nonionic (low and isoosmolar) agents. Although the introduction of nonionic ICMs reduced the incidence of AARs in ICM, the incidence of AARs to nonionic ICMs continue to occur compared to the previous ionic ICM, and significant medical issue presented occasionally, especially in cases of severe AARs [6]. It was reported that the incidence of AARs to nonionic ICM ranges widely from 0.2% to 3% [7,8].

Currently, despite the generalized use of ICM, immediate hypersensitivity reactions to their widespread use remain a common clinical concern, and the mechanism underlying these reactions is still unclear in many cases. Cutaneous mild reactions are the most common symptom of immediate hypersensitivity reactions (IHRs) to ICMs, but severe anaphylaxis or even death can occur with an estimated death rate of 1 in 100,000 examinations [9]. Hypersensitivity reactions may occur *via* immunologic or non-immunologic mechanisms [10], immunologic anaphylaxis can be divided into IgE-dependent and immunoglobulin E (IgE)-independent mechanism, whereas IgE-

Abbreviations: AARs, acute adverse reactions; HOCM, high osmolar contrast media; ICM, iodinated contrast media; IgE, immunoglobulin E; IHRs, immediate hypersensitivity reactions; Kit mice, mast cell-deficient W-sash c-kit mutant $Kit^{W\text{-}sh/W\text{-}sh}$ mice; LOCM, low osmolar contrast media; MCs, mast cells; MCDM, mast cell dissociation media; MRGPRX2/B2, MAS-related G proteincoupled receptor-X2/B2; MPMCs, mice peritoneal mast cells; MUT mice, MrgprB2 mutant mice; NC, negative control; PGD2, prostaglandin D2; SCF, stem cell factor; siRNAs, small interfering RNAs; TNF-α, tumor necrosis factor-α; WT, wild-type

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independent anaphylaxis involves direct mast cell (MC) activation [11]. ICM induced anaphylaxis reactions could also be divided into IgE-dependent or independent manner [12,13]. The symptoms of IgE-independent anaphylaxis to ICM were mainly caused by non-specific direct histamine release from MCs and basophils [14,15]. However, it has not been reported about the specific mechanism of ICM-induced MC related anaphylactoid reactions.

Recently, it was proposed that MAS-related G proteincoupled receptor-X2 (MRGPRX2) mediated the IgE-independent anaphylaxis involving specific drugs, such as icatibant, quinolone antibiotics and neuromuscular blocking drugs [16]. These drugs can stimulate MCs degranulation and release tumor necrosis factor-α (TNF-α) and prostaglandin D2 (PGD2), leading to IgE-independent anaphylactoid reactions [17]. It has been reported that medications such as non-steroidal anti-inflammatory drugs (NSAIDs), beta-lactam antibiotics, quinolones, vancomycin, and opioids could stimulate MCs through MRGPRX2 directly, and leading to anaphylactoid reactions [10,11,17]. MrgprB2 is the mouse counterpart of MRGPRX2, which also participates in drug-induced anaphylactoid reactions in mice [16].

Iopamidol, one of the commonly nonionic ICM, is used for improving imaging resolution and usually at high concentrations. The occurrence rate of overall AAR incidence after using Iopamidol was reported to be 1.21% [18]. Jeffrey S. et al. reported [19] that 15 ml of Iopamidol (300 mg/ml) was applied during imageology examination, and J. Valk et al. also gave iopamidol for 300 mg/ml [20]. Douglas H. et al. reported even though low concentration of iopamidol (30 mg/ml) in stock, IV injection speed (5 ml/s) seemed result in high blood concentration [21]. Therefore, we selected different concentrations of iopamidol based on the previous studies and our preliminary experiment. The main purpose of the study is to investigate the mechanism of Iopamidol induced anaphylactoid reactions *via* MRGPRX2. Also, we want to provide first report and ideas for ICM triggered anaphylactoid reactions.

2. Materials and methods

2.1. Drugs and reagents

Compound 48/80, Evans blue and *p*-nitrophenyl *N*-acetyl-β-D-glucosamide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Iopamidol was purchased from Dalian Meilun Biotechnology Co., LTD (Dalian, Liaoning, China) and purified to ≥99%. Saline was purchased from Shandong Qilu Pharmaceutical Co., Ltd. (Jinan, Shandong, China). A 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 background MrgprB2-knockout (MUT) mice were kindly provided by Professor Xinzhong Dong from Johns Hopkins University (Baltimore, MD, USA), and $Kit^{W\text{-}sh/W\text{-}sh}$ mice on C57BL/6 back ground were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). Wild-type (WT) C57BL/6 mice were raised in the Experimental Animal Center at Xi'an Jiaotong University (Xi'an, China). The mice aged 6–8 weeks were used in all the experiments in this study. Animals were housed at the Experimental Animal Center of Xi'an Jiaotong University and provided food and water adlibitum.

2.3. Ethics statement

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: XJTULAC2019-1021).

2.4. Hindpaw swelling and extravasation assay

Young adult mice (aged 6–8 weeks old) were anesthetized with 70 mg/kg pentobarbital sodium by intraperitoneal injection. Fifteen minutes after anesthesia induction, each mouse was intravenously (*i.v.*) injected with 50 μl 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 μl of 75 mg/ml, 150 mg/ml, or 300 mg/ml Iopamidol or 10 μg/ml C48/80 was injected into the left paw, and saline was injected into the right paw as a negative control. Another fifteen minutes later, the paw thicknesses were measured again. Mice were then euthanized by decapitation, and the paw tissues were collected, dried at 50 °C, and then weighed separately. Evans blue dye was extracted by adding 500 μl of a mixture of acetone-saline (7,3, v/v) to each tissue sample and incubating at 37 °C for 12 h. Thees tissues were then minced, and disrupted for 10 min in an ultrasonic machine, and centrifuged at 5000 rpm for 10 min. The supernatant was aliquoted equally into 96-well plates (200 μl/well), and the OD value at 620 nm was measured using a Microplate Reader.

2.5. Mice peritoneal mast cell purification assay

Young adult mice (8–10 weeks of age) were euthanized *via* excessive CO_2 inhalation. A total of 12 ml of ice-cold MCs dissociation media (MCDM; HBSS with 3% FBS and 10 mM HEPES, pH 7.2) was used to make two sequential peritoneal lavages, which were then collected and centrifuged at 200 ×*g* and 4 °C for 5 min. The cells from each mouse were resuspended in 2 ml MCDM, layered over with 4 ml of isotonic 70% Percoll suspension (2.8 ml of Percoll, 320 μl of 10 × HBSS, 40 μl of 1 M HEPES, 830 μl of MCDM), and centrifuged at 500 ×*g* and 4 °C for 20 min. The pellet was MCs and the purity was > 95% as assayed by morphology and toluidine blue staining. MCs 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 (SCF) and seeded into 96-well plates for the follow-up experiments.

2.6. Cell lines

Laboratory of Allergic Disease 2 (LAD2) human MCs were kindly provided by A. Kirshenbaum and D. Metcalfe from NIH (Bethesda, MD, USA). Cells were maintained in StemPro-34 medium supplemented with StemPro nutrient supplement, 100 U/ml of penicillin-streptomycin, 2 mM L-glutamine and 100 ng/ml human stem cell factor in a 37 °C humidified incubator containing 5% CO_2. Cell culture medium was replaced every 7 days, and the cells were maintained at a density of 2×10^6 cells/ml. HEK293-MRGPRX2 cells were kindly provided by Professor Xinzhong Dong from Johns Hopkins University (Baltimore, MD, USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml of penicillin-streptomycin (HyClone, UT).

2.7. The β-hexosaminidase release assay

MCs were seeded into a 96 well plate at 2×10^4 cells per well and incubated for 2 h. Then, the culture medium was removed, either Iopamidol or C48/80 at the indicated concentrations diluted in modified Tyrode's solution (120 mM NaCl, 2.5 mM $CaCl_2$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 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 200 ×*g* 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 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. MCs were seeded into a 96 well plate at 2×10^4 cells per well and incubated for 2 h. Then, the culture medium was removed, either Iopamidol or C48/80 at the indicated concentrations diluted in modified Tyrode's solution was added to the wells and incubated for 30 min. The 96-well plate was centrifuged at 200 ×*g* for 5 min. The supernate was collected and measured the level of histamine.

2.9. Measurements of tumor necrosis factor (TNF)-α levels

The levels of TNF-α in cell supernatant were detected using human TNF-α enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions. After cells were incubated with Iopamidol, C48/80 or vehicle for 12 h, the levels of TNF-α was measured in modified Tyrode's solution using commercially available ELISA kits designed for it according to the manufacturers' instructions.

2.10. Intracellular Ca^{2+} mobilization assay

Either Iopamidol or C48/80 at the indicated concentrations 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, 1.2 mM $NaHCO_3$, 20 mM glucose, 20 mM sucrose, brought to pH 7.4 using NaOH). For imaging, the cells were washed twice in CIB, and The incubated with 4 μM Fluo-3 AM buffer in CIB. Fluo-3 AM loaded cells were imaged at excitation wavelengths of 488 nm, respectively. Unless otherwise specified, drugs were added to the well at 10 s after initial imaging, and responses were continuous monitored for an additional 120 s.

2.11. 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 were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences were as follows: Negative Control (NC), forward, 5′-UUCUCCGAACGUGUCACGUTT-3′, and reverse, 5′-ACGUGACACGUUCGGAGAATT-3′; MRGPRX2, forward, 5′-GUACAACAGUGAAUGGAAATT-3′, and reverse, 5′-UUUCCAUUCAC UGUUGUACTT-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.

2.12. Reverse-transcriptase PCR analysis

Total RNA from the transfected LAD2 cells was isolated using a Total RNA Extraction kit (Takara). Total RNA was then reverse-transcribed in 20 ml reaction solution (containing 4 μl total RNA and 16 μl PCR mixture) using a Revert AID™ First Strand cDNA Synthesis kit (Takara). The cDNA was synthe-sized using Bio-Rad iScript Reverse Transcriptase (Bio-RAD) and the PCR reactions were performed using a Thermal Cycler Dice Real Time system (Takara). The primer sequences were as follows: GAPDH forward, 5′-CACCCACTCCTCCACCTTTG-3′ and reverse, 5′-CCACCACCCTGTTGCTGTAG-3′, MRGPRX2 forward, 5′-CGATCCTCAAGCTGGCTCTC-3′, and reverse, 5′-GTCCATCTCTACAC CAGACTGCTTC -3′, synthesised by Shanghai GenePharma Co., Ltd. The thermocycling conditions were as follows: 95 °C for 2 min and then 95 °C for 10 s, and 45 cycles of 60 °C for 20 s. The relative level of MRGPRX2 mRNA for were normalized and represented as the ratio of the GAPDH gene.

2.13. Western blot analysis

Total protein in siRNA transfected LAD2 cells was extracted on ice for 30 min using RIPA lysis buffer containing 10% protease inhibitor cocktail (Roche Diagnostics). Insoluble protein lysate was removed by centrifuging the samples at 13,500 × g for 10 min at 4 °C. The protein concentration was determined using a BCA Protein Quantification kit according 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 primary antibodies of anti-MRGPRX2 (1:1000, #ab237947, abcam) 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.

2.14. Statistical analysis

The group data are expressed as the mean ± S.E.M. Independent sample variance analysis was used to determine the significance of statistical comparisons using SPSS software. The data was analyzed using the Mann-Whitney test or Kruskal-Wallis test. Individual differences between treatment and control groups were identified using Dunn's test. Differences were considered significant at $p < 0.05$ ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

3.1. Iopamidol-induced anaphylactoid reactions are MrgprB2-dependent in mice

A mouse model of passive cutaneous anaphylaxis was used to explore the *in vivo* Iopamidol-induced anaphylactoid reaction. The results showed that although subcutaneous injection of saline (vehicle) did not induce hindpaw inflammation (swelling and extravasation), Iopamidol injection evoked extensive extravasation and swelling in mice in a dose-dependent manner (Fig. 1). Compound 48/80, a classical mast cell activator and canonical basic secretagogue, was used as positive control. We next tested whether the development of hindpaw inflammation

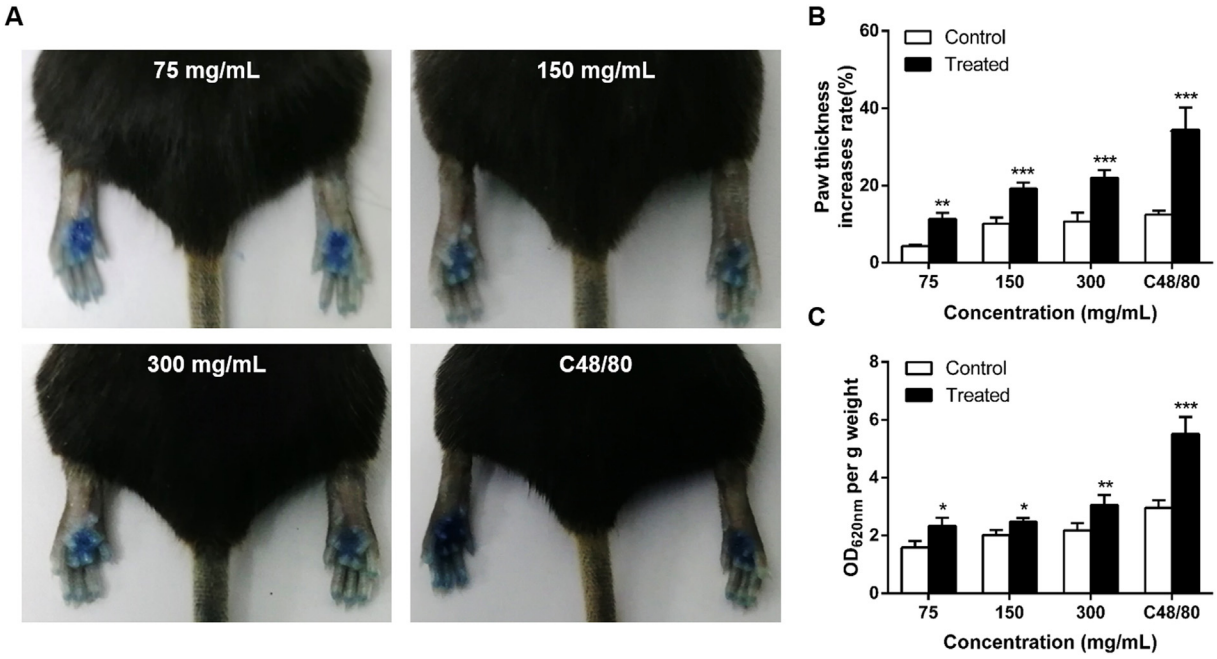

Fig. 1. Iopamidol-induced cutaneous flare reactions were dose-dependent in mice. (A) Representative images of Evans blue stained extravasation 15 min after intraplantar injection of 5 µl 75 mg/ml, 150 mg/ml, 300 mg/ml Iopamidol or 10 µg/ml C48/80 in the left paw of C57BL/6 mice, or saline in the right paw. (B) Quantification of paw thickness increase 15 min after intraplantar injection. (C) Quantification of Evans blue leakage into the paw after 15 min after intraplantar injection. ($n = 6$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

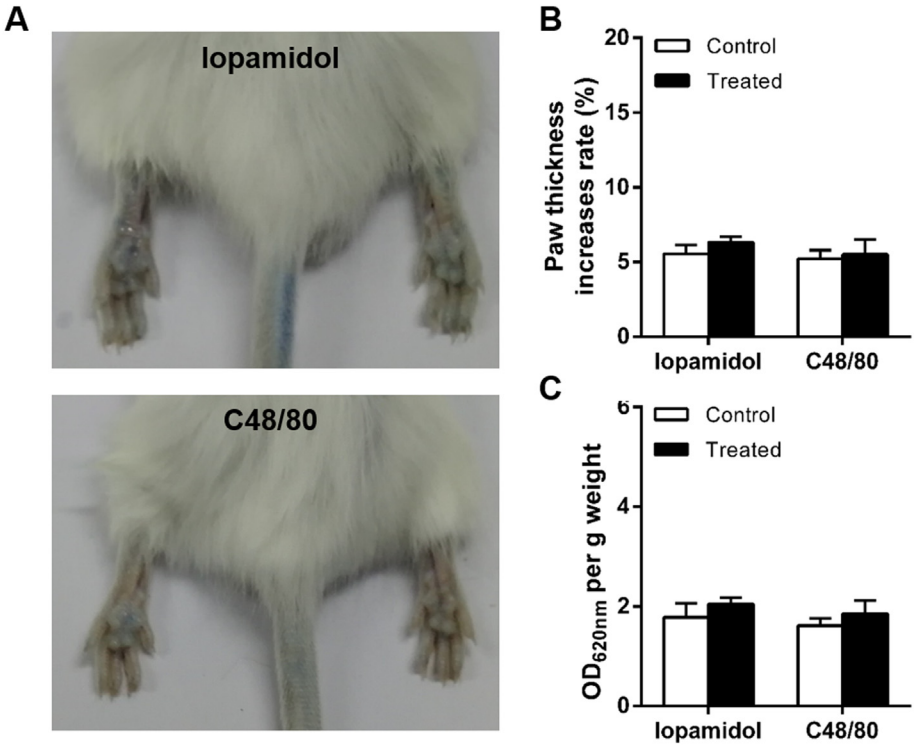

Fig. 2. Iopamidol-induced cutaneous flare reactions were associated with mast cells. (A) Representative images of Evans blue stained extravasation 15 min after intraplantar injection of 5 µl 300 mg/ml Iopamidol or 10 µg/ml C48/80 in the left paw of $Kit^{W\text{-}sh/W\text{-}sh}$ mice, or saline in the right paw. (B) Quantification of paw thickness increase 15 min after intraplantar injection. (C) Quantification of Evans blue leakage into the paw after 15 min after intraplantar injection. (n = 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

after Iopamidol injection was associated with MCs using a C57BL/6 background $Kit^{W\text{-}sh/W\text{-}sh}$ mice model. We observed that Iopamidol-induced hindpaw inflammation was almost completely absent in C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice (Fig. 2), which indicates that the anaphylactoid reactions caused by Iopamidol were associated with MCs. MrgprB2 mutation (MUT) mice were used to investigate whether Iopamidol-induced hindpaw inflammation was associated with MrgprB2 expression on MCs, and we found that compared to WT mice, MUT mice exhibited almost no hindpaw inflammation (Fig. 3), which suggests that the *in vivo* activation of mast cell mediator release and the subsequent Iopamidol-induced vasodilation was MrgprB2-dependent.

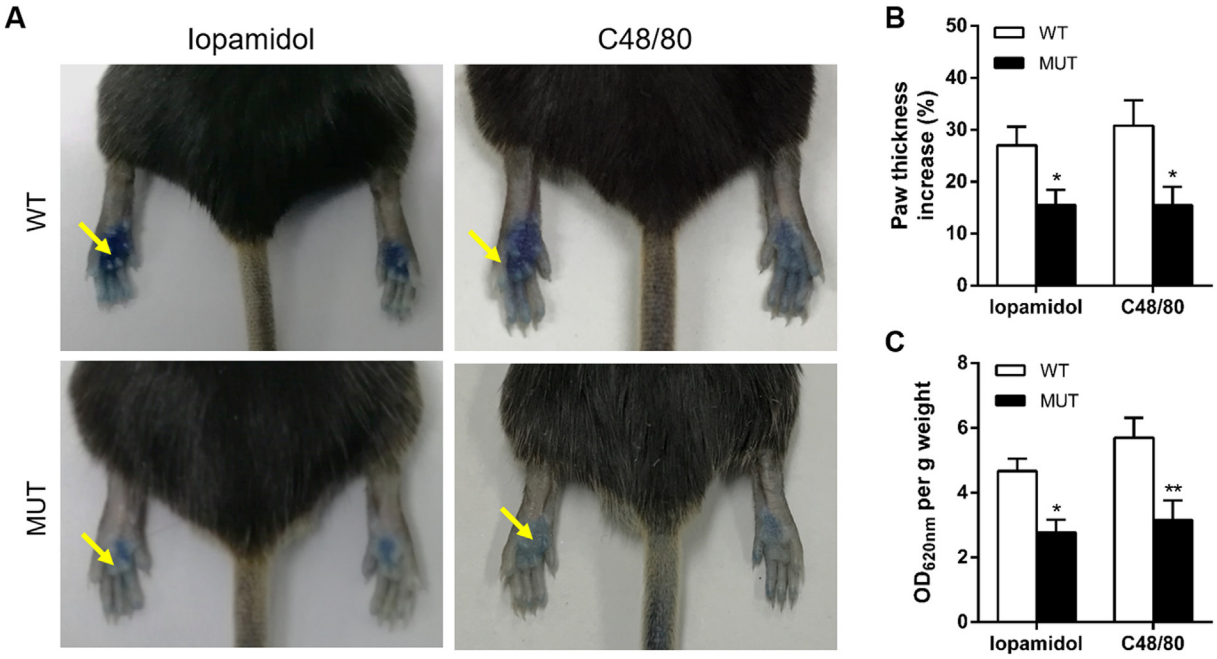

Fig. 3. MrgprB2 played an important role during Iopamidol-induced cutaneous flare reactions. (A) Representative images of Evans blue stained extravasation 15 min after intraplantar injection of 5 μl 300 mg/ml Iopamidol or 10 μg/ml C48/80 in the left paw of WT mice and MrgprB2 MUT mice, or saline in the right paw. (B) Quantification of paw thickness increase after 15 min. (C) Quantification of Evans blue leakage into the paw after 15 min. (n = 6, $^{**}p < 0.01$, $^{***}p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. MrgprB2 is crucial for activation of MPMCs

Mice peritoneal mast cells (MPMCs) are connective tissue MCs. Calcium imaging of MPMCs showed a significantly increased intracellular calcium mobilization of WT mice treatment with 300 mg/ml Iopamidol (Fig. 4A). Under the treatment of 75, 150, 300 mg/ml Iopamidol, the β-hexosaminidase release showed a promoted secretion dose-dependently (Fig. 4B). The MPMCs also showed increased release of histamine treated by 75, 150, 300 mg/ml Iopamidol (Fig. 4C). Compound 48/80 was used as positive control. MrgprB2 expression has been reported to be highly specific to connective tissue MCs, which is a target of many small molecule drugs associated with anaphylactoid reactions. The calcium imaging results indicated that treatment with 300 mg/ml Iopamidol in MrgprB2 MUT MCs was almost no responding to calcium mobilization (Fig. 4D). Meanwhile, there was no significant increase of β-hexosaminidase or histamine release treated by the same

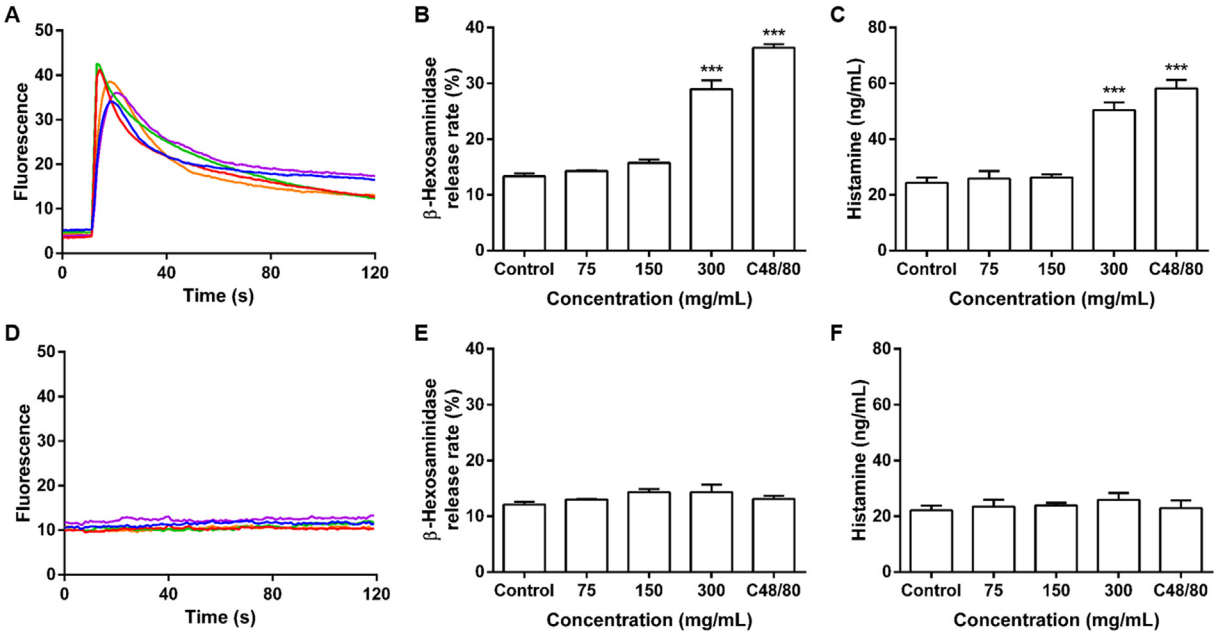

Fig. 4. Iopamidol-induced activation of MPMCs. (A) Calcium imaging of WT MPMCs triggered by 300 mg/ml Iopamidol. (B) The β-hexasaminidase release in WT MPMCs treated with Iopamidol for 30 min. (C) The histamine release in WT MPMCs treated with Iopamidol for 30 min. (D) Calcium imaging of MUT MPMCs triggered by 300 mg/ml Iopamidol. (E) The β-hexasaminidase release in MUT MPMCs treated with Iopamidol for 30 min. (F) The histamine release in MUT MPMCs treated with Iopamidol for 30 min. ($n = 5$, $^{***}p < 0.01$).

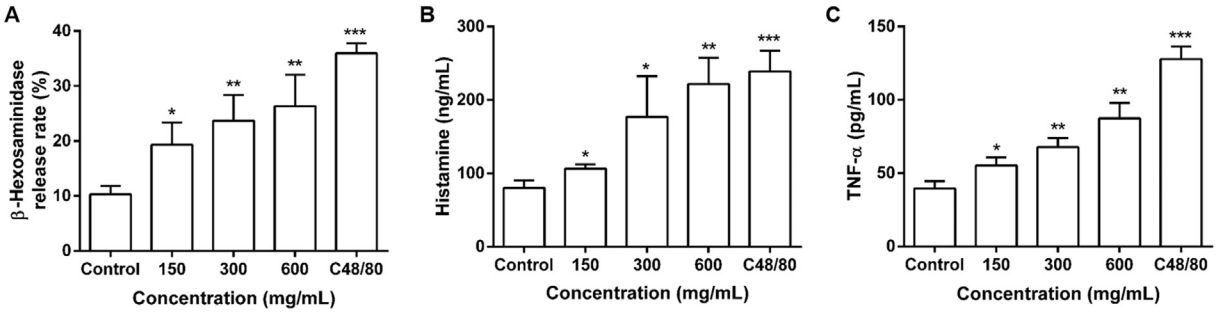

Fig. 5. Iopamidol-induced degranulation of LAD2 mast cells. (A) The β-hexasaminidase release in LAD2 cells treated with Iopamidol for 30 mins. (B) The histamine release in LAD2 cells treated with Iopamidol for 30 mins. (C) The TNF-α release in LAD2 cells treated with Iopamidol for 6 h. (n = 5, $*p < 0.05$, $**p < 0.01$, $***p < 0.01$).

concentration of Iopamidol (Fig. 4E, F). Therefore, MrgprB2 played an important role during Iopamido-induced activation of MPMCs.

3.3. Iopamidol-induced mast cell degranulation

To verify whether the Iopamidol-induced activation of MCs promoted degranulation, we LAD2 mast cell line was used as a model to detect changes in the secretion of β-hexosaminidase, histamine and TNF-α, the latter two of which are mediators of increased vascular permeability. LAD2 MCs were treated with different concentrations of Iopamidol (0, 150, 300, 600 mg/ml) for 30 min, the results showed a dose-dependently increase of β-hexosaminidase and histamine (Fig. 5A, B). After 6 h's treatment of Iopamidol (0, 150, 300, 600 mg/ml), the secretion of TNF-α from LAD2 cells was also promoted by a dose-dependent manner (Fig. 5C). 10 μg/ml Compound 48/80 was used as positive control. Hence, Iopamidol has the ability to trigger mast cell degranulation, and then induce anaphylactoid reactions.

3.4. MRGPRX2 mediates Iopamidol-induced mast cell activation during IgE-independent anaphylactoid reactions

MRGPRX2 knockdown LAD2 cells was performed to verify the role of MRGPRX2 during Iopamidol-induced non IgE-dependent anaphylactoid reactions. The MRGPRX2 siRNA transfection effect was measured by the reverse-transcriptase PCR analysis and western blot analysis (Fig. 6A, B, C), which showed a significant reduction of MRGPRX2 level. Under the treatment of 0, 150, 300, 600 mg/ml Iopamidol in LAD2 cells, the levels of secreted β-hexosaminidase, histamine and TNF-α were measured, 10 μg/ml C48/80 was used as a positive control. The results reflected that the degranulation of β-hexosaminidase, histamine and TNF-α were significantly reduced when knockdown the MRGPRX2 level (Fig. 6D, E, F). Calcium imaging analysis of MRGPRX2/HEK293 cells or HEK293 cells treated by 300 mg/ml Iopamidol showed that Iopamidol triggered higher intracellular Ca^{2+} levels in MRGPRX2/HEK293 cells, almost no effect on HEK293 cells (Fig. 7). Hence, MRGPRX2 mediated the Iopamidol-induced mast cell activation.

4. Discussion

This experiments demonstrated that a mast cell specific receptor, MRGPRX2, mediated Iopamidol triggered MCs calcium mobilization and degranulation, and evoked the IgE-independent anaphylactoid reactions. It has been assumed that immediate, anaphylaxis-like reactions caused by ICM were relied on mast cell membrane leading to mediator release or by direct complement activation possibly [22]. MCs store many biological activities and immunomodulatory molecules, such as chemokines, cytokines, histamine, serotonin, and tryptase [23]. When activation, the related substance released to the tissue environment that can impact processes as varied as tissue remodeling, vascular tone and immune response [24]. MRGPRX2 is responsible for these reactions that is confined to MC_{TC} type MCs, basically the only human skin MCs subset [25,26]. In this experiment, MCs calcium imaging and release of β-hexosaminidase, histamine and TNF-α were detected, which showed a marked calcium mobilization and degranulation stimulated by Iopamidol in a dose-dependent manner. Meanwhile, knockdown of MRGPRX2 in LAD2 cells reflected a reduction of degranulation. Iopamidol also has the ability to activate the MRGPRX2/HEK293 cells but has no effect on MRGPRX2/HEK293 cells. Combine the results from animal experiments that MrgprB2 mediated Iopamidol induced hindpaw swelling and extravasation in mice, we concluded that MRGPRX2/B2 played a decisive role in the anaphylactoid reaction caused by Iopamidol.

The risk for developing immediate or delayed hypersensitivity reactions to ICM interferes with the diagnosis and treatment of a number of patients requiring imaging diagnostic methods for many common diseases. ICM induced adverse reactions were unpreventable and unavoidable from the dose when appropriately used and should be paid more attention to their safe use [27]. Due to the chemical structures, route of administration, and the non-adjustability of the administration dose, the mechanisms of contrast agents induced adverse reactions were still a matter of debate, more in depth investigation and further pharmacovigilance studies are required. The osmolarity of ICM is an obvious risk factor for hypersensitivity. High osmolar contrast media (HOCM) have been replaced by low osmolar contrast media (LOCM) since the late 1980s, which made the incidence of severe immediate reactions reduce up to 10-fold [28,29]. Iopamidol is a widely used ICM for diagnosis with a 13.9% incidence of adverse reactions [30]. Iopamidol and other iodine contrast agent induced anaphylactoid reactions include erythema, urticaria, generalized skin rash, pulmonary oedema, dyspnoea, coughing, sneezing of respiratory system, and nausea, vomiting and dry mouth gastrointestinal tract, respiratory, cutaneous and cardiovascular symptoms [30].

The study of the mechanism of Iopamidol triggered anaphylactoid reactions *via* MRGPRX2 can further explain the reason of ICM induced immediate IgE-independent anaphylactoid reactions. The ligands targeting to MRGPRX2 are numerous, which comprise Compound 48/80, drugs like neuromuscular blocking agents, fluoroquinolones, host defense peptides and neuropeptides like SP [16,31]. This experiment expanded its ligand range to ICM for the first time. The role of MRGPRX2 in ICM-induced anaphylactoid reactions has not been reported before. Addressing this issue would enable doctors and researchers to better understand the mechanisms of immediate ICM-induced reactions and may improve patient safety in the long term. The mast cell degranulation triggered by Iopamidol was dose-dependent, therefore, the higher the Iopamidol dose used, the greater risk and severity of anaphylactoid reactions may occur. The correlation between ICM concentrations eliciting skin response and the expression of MRGPRX2 in the skin can be assessed, together with investigation of polymorphisms in the MRGPRX2 gene, which may affect the receptor function, especially in the individuals presenting extreme responses in skin tests

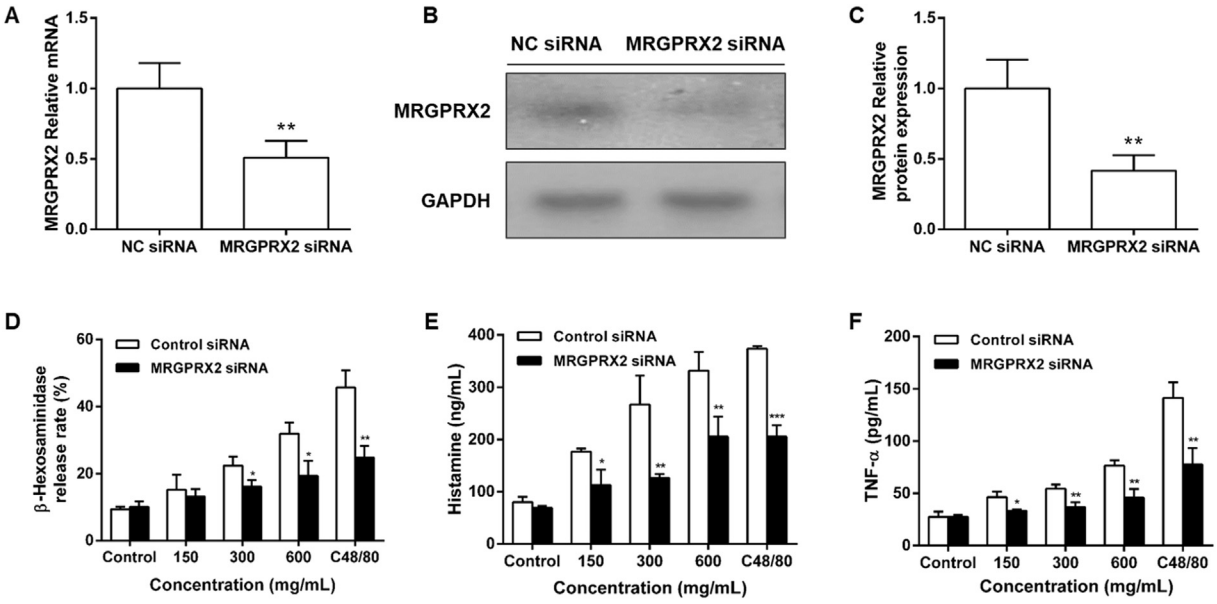

Fig. 6. The degranulation of mast cell triggered by Iopamidol *via* MRGPRX2. (A) The MRGPRX2 mRNA expression in LAD2 cells transfected with 1 μM Negative Control (NC), or MRGPRX2 siRNA respectively using Lipofectamine® 2000. (B) Protein expression levels of MRGPRX2 in the LAD2 cells transfected with siRNA. (C) Quantification of MRGPRX2 protein expression by densitometric analysis. (D) The β-hexasaminidase release in transfected MRGPRX2 knockdown LAD2 cells treated with Iopamidol for 30 mins. (E) The histamine release in transfected MRGPRX2 knockdown LAD2 cells treated with Iopamidol for 30 mins. (F) The TNF-α release in transfected MRGPRX2 knockdown LAD2 cells treated with Iopamidol for 6 h ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$).

(responding to the highest and the lowest drug concentrations).

Mouse MrgprB2 is the homologous gene of human MRGPRX2. MRGPRX2 has been reported to be a unique receptor important for modulating mast cell degranulation and bind to many well-known synthetic cationic agonists [17,32]. Based on structure analysis, there are three cationic nitrogen atoms in Iopamidol, which is important for MRGPRX2 activation. Therefore, we argue that Iopamidol bind to MRGPRX2, leading to the activation of the relevant signaling molecules and calcium signal, and then releasing of the sensitization matter. Further confirmation requires more experiments about the binding effect and signal pathways, and clinical study of the Iopamidol hypersensitivity patients, including the skin test, basophil activation test and quantification of specific IgE antibodies to Iopamidol.

The mechanism of ICM-induced anaphylactoid reactions are complex, involving complement activation, modulation of enzymes and proteolytic cascades in plasma, and direct degranulation of mast cells and basophils [33]. In this study, particular attention was paid to the evidence supporting a mast cell receptor activation as an underlying cause of a typical ICM, Iopamidol, induced anaphylactoid reactions *in vitro and in vivo*. With the evidence indicating underlying the allergic mechanisms, studies regarding the skin tests using ICM were necessary, and the development of novel MRGPRX2 antagonists would bring a reduction of ICM-induced anaphylactoid reactions in clinical.

5. Conclusions

In conclusion, this study demonstrated that Iopamidol stimulates mouse MrgprB2 and promote mast cell degranulation, which is essential for Iopamidol induced anaphylactoid reactions in wild type mice. MRGPRX2/B2 mediated Iopamidol triggered human mast cell release of anaphylactic mediators and intracellular calcium responses. This study elucidated a foundation for the pre-clinical development of ICM-induced anaphylactoid reactions using Iopamidol as an initial candidate compound.

Declaration of competing interest

The authors declare no financial or commercial conflict of interest.

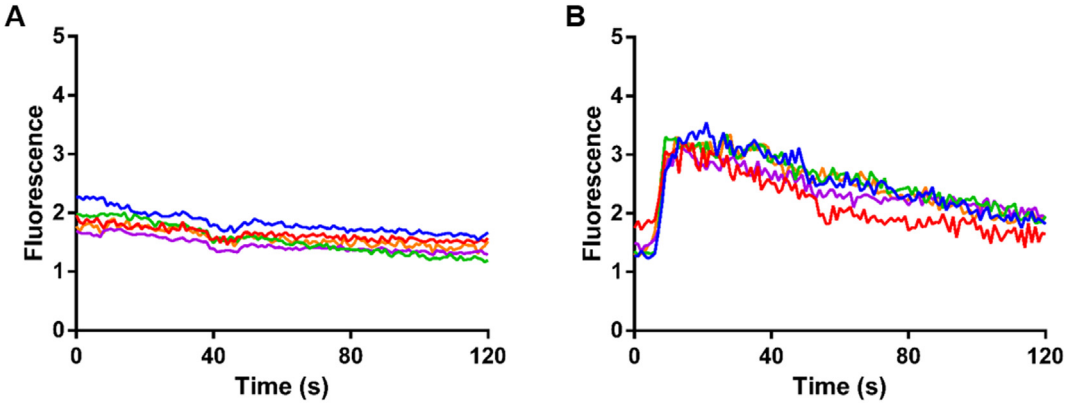

Fig. 7. The activation of MRGPRX2 triggered by Iopamidol. (A) Representative imaging traces of Ca^{2+} concentrations treated by 300 mg/ml Iopamidol in NC/HEK293 cells. (B) Representative imaging traces of Ca^{2+} concentrations treated by 300 mg/ml Iopamidol in MRGPRX2/HEK293 cells.

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