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Gold induces a pseudo-allergic reaction via MRGPRX2 both *in vitro* and *in vivo*

Yingnan Zeng[a], Jue Wang[a], Yongjing Zhang[a], Shuai Ge[a], Yuanyuan Wu[b], Ting Fan[c], Nan Wang[a,*]

[a] College of Pharmacy, Xi'an Jiaotong University, Xi'an 710061, China
[b] Department of Respiratory and Critical Care Medicine, The Second Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710004, China
[c] Department of Pharmacy, Hong Hui Hospital, Xi'an Jiaotong University College of Medicine, Xi'an 710054, China

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ABSTRACT

The traditional mast cell (MC) degranulation pathway is mediated by crossing-linking of high-affinity IgE receptor (FcεRI), whereas a non-traditional, but analogous, pseudo-allergic way was recently reported to occur via Mas-Related G Protein-Coupled Receptor X2 (MRGPRX2). Severe contact hypersensitivity to metallic gold, typically considered non-sensitizing, has been reported. However, whether gold induces IgE-independent allergy remains unclear. Therefore, this study assessed the effects of gold chloride (CA) on MC activation and its relation to MRGPRX2. Our data show that CA acted on MRGPRX2 to increase cellular calcium levels and induced the release of inflammatory mediators *in vitro*. Compared to Mrgprb2-knockout (KO) mice, CA dose-dependently induced passive cutaneous anaphylaxis (PCA) in wild-type (WT) mice. Furthermore, peritoneal mast cells (MPMCs) were extracted from WT and Mrgprb2-KO mice and stimulated by CA, but only MPMCs from WT mice could be activated. Our results suggest that CA-induced pseudo-allergic responses are MRGPRX2 dependent.

1. Introduction

Metallic gold, including jewelry gold and other gold alloys, has been widely accepted as a non-sensitizing material [1]. Contact dermatitis to gold is therefore considered to be rare, as allergies to metallic gold have traditionally been difficult to demonstrate. However, Kligman found CA to be a strong sensitizer in a human maximization test [2]. Furthermore, wearing gold in pierced ears, mechanical abrasion of gold jewelry, and corrosion of gold induced by certain components of sweat can also lead to contact dermatitis [3].

MCs are the primary mediators in allergic reactions. They are principally located in the skin, respiratory tract, oral/gastrointestinal mucosa and other exterior environments [4]. MCs play important roles in allergies and may be activated via two different pathways. Classically, they are activated as a result of cross-linking between antigens and surface-bound IgE, via FcεRI [5]. However, MCs can also be activated via MRGPRX2 and show IgE-independent responsiveness in this context [6].

Human MRGPRX2 is homologous to mouse Mrgprb2, and these receptors are associated with pseudo-allergic reactions [6]. There is evident that MRGPRX2 is a target receptor for a variety of drugs [7–9] and that is drug-mediated activation subsequently induces calcium influx, MC degranulation, and release of pro-inflammatory mediators. Many drugs induce pseudo-allergic reactions through the MRGPRX2-induced MC activation [10].

It has reported that gold can sensitize BN rats to IgE-mediated degranulation and IL-4 transcription and secretion [11]. Hayama et al. studied the mechanisms of gold-induced mast cell activation, comparing them with IgE-dependent activation mechanisms [12]. Gold-induced IgE-dependent allergies have been studied extensively. However, investigation of gold induction in IgE-independent allergies has not been reported. The aim of this study was to determining the effects of gold on MC activation and its relation to MRGPRX2.

2. Materials and methods

2.1. Drugs and reagents

CA was provided by Shanghai Fine Chemical Materials Research Institute (Shanghai, China). Human TNF-α and IL-8 ELISA Kit were purchased from ExCell Biology, Inc. (Shanghai, China). Histamine, Evans blue and Compound 48/80 (C48/80) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluo-3, AM ester was obtained from Biotium (Waltham, MA, USA). StemPro-34 medium and human stem cell factor were purchased from Cell Signaling Technology (Danvers, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and

* Corresponding author at: School of Pharmacy, Xi'an Jiaotong University, Yanta West Road, Xi'an 710061, China.
E-mail address: wangnan2014@xjtu.edu.cn (N. Wang).

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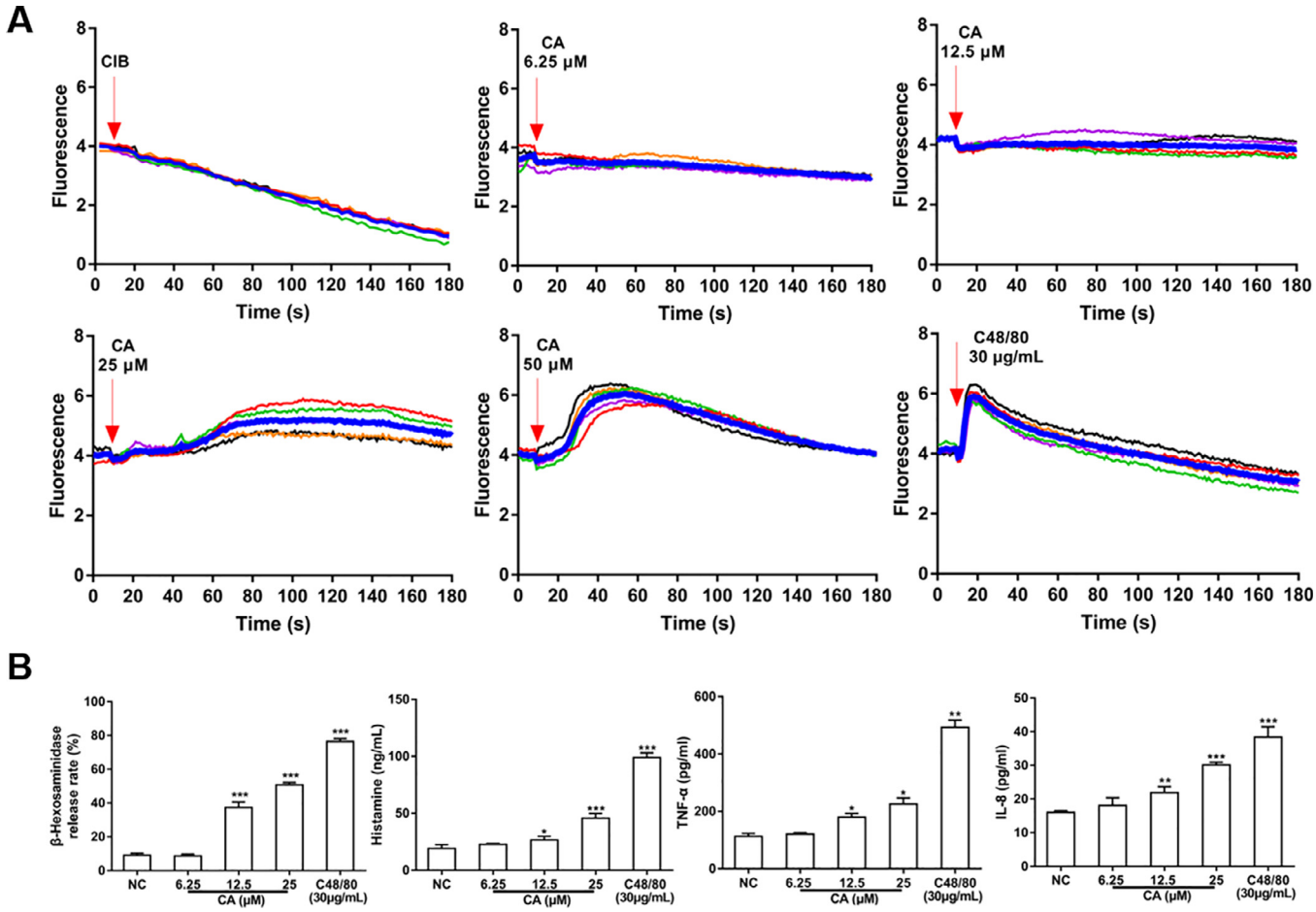

Fig. 1. CA induced mast cell degranulation. (A) CA increased intracellular calcium concentrations in LAD2 cells in a dose-dependent manner. CA was added at 10 s and each thick blue line represents the mean change of fluorescence at this concentration. (B) β-hexosaminidase, histamine, TNF-α, and IL-8 release from LAD2 cells treated with different concentrations of CA (6.25, 12.5, and 25 μM). Experiments were repeated 3 times. Data are expressed as mean ± SD and were analyzed using two-tailed unpaired Student's t-tests. Differences were considered significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

penicillin-streptomycin were purchased from Hyclone (Chicago, IL, USA). Chloral hydrate was sourced from HUSHI (Shanghai, China). *E. coli*, containing the MRGPRX2 plasmid was provided by VectorBuilder (Shenandoah, TX, USA). The Endo-Free Plasmid Mini Kit I was purchased from Omega Bio-tek (Norcross, GA, USA). BD IMag Magnetic Positive Marking and Sorting Kit was purchased from BD Biosciences (San Jose, CA, USA).

2.2. Animals

Adult male C57BL/6 mice (6–8 weeks) were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Mrgprb2-knockout (KO) mice in a C57BL/6 background were kindly provided by Professor Xinzhong Dong from Johns Hopkins University (MD, USA). All mice were housed with a constant temperature of 20–25 °C and in a specific-pathogen-free environment, with free access to food and water.

2.3. Ethical considerations

This study was conducted in strict accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH). Experimental protocols involving mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an China (Permit number: XJTU 2011-0045).

2.4. Cell lines

LAD2 human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, MD, USA). Cells were maintained in StemPro-34 medium supplemented with 100 U penicillin-streptomycin, 2 mM L-glutamine, and 100 ng/mL human stem cell factor in a 37 °C incubator with 5% CO_2.

MRGPRX2-expressing HEK293 cells were constructed in our laboratory using plasmid transfection. Mrgprb2-HEK293 cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH). Cells were cultured in high glucose DMEM with 100 U penicillin-streptomycin and 10% fetal bovine serum (FBS).

2.5. Intracellular calcium image assay

All drugs used in these experiments were diluted to the required concentration in calcium imaging buffer (CIB: 125 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 0.6 mM $MgCl_2$, 10 mM HEPES, 20 mM glucose, 1.2 mM $NaHCO_3$, 20 mM sucrose, adjusted to pH 7.4 using NaOH). Cells were incubated at 37 °C for 30 min with 0.1% Fluo-3, AM easter. For imaging, cells were washed twice with CIB and imaged at 488 nm excitation. Unless otherwise specified, drugs were added to the wells 10 s after the initial imaging and images were captured at 1-s intervals for an additional 170 s.

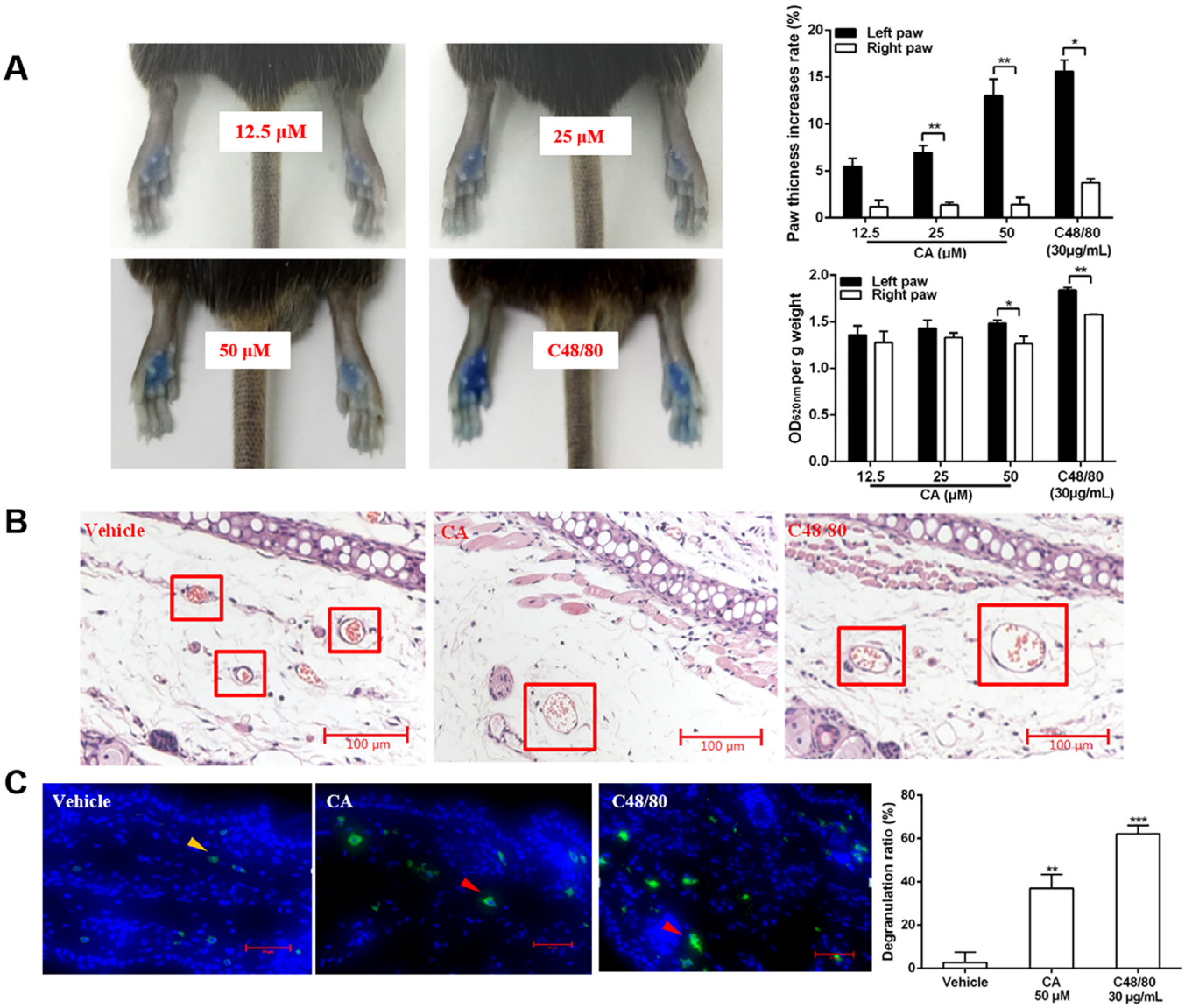

Fig. 2. CA induced pseudo-allergy by increasing mast cell degranulation in mice. (n = 5 mice per group; experiments were repeated > 3 times; data are presented as the mean ± SD and were analyzed by two-tailed unpaired Student's *t*-test). (A) CA induced PCA in C57 WT mice. CA was administered at concentrations of 12.5, 25, and 50 μM, using a microinjector, into the left paw; saline was administered in the same manner to the right paw as a negative control. Histograms show quantification of paw thickness and Evans blue leakage into the paw after 15 min. Differences were considered significant at $^{*}p < 0.05$ and $^{**}p < 0.01$. (B) CA induced hemangiectasis as demonstrated by HE staining of skin tissue sections. (C) Avidin staining of ear skin after treatment with vehicle, CA, or C48/80. Yellow arrow indicates a normal MC. Red arrow indicates a degranulated MC. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle.

2.6. β-Hexosaminidase release assay

LAD2 cells were seeded into a 96-well plate at 2×10^5 cells per well and incubated for 2 h at 37 °C. The culture medium was removed and drugs were diluted to the indicated concentrations in TM buffer (120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, 5.5 mM glucose, 5 mM bovine serum albumin) and added to the wells. The cells were then incubated at 37 °C for 30 min; supernatants were subsequently collected and cells were lysed with 0.1% Triton X-100 in TM buffer. β-hexosaminidase quantified in supernants and lysates by monitoring 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. Reactions were terminated by adding stop buffer (0.1 M sodium carbonate/sodium bicarbonate, pH 11.0). The percentage of β-hexosaminidase released was assessed by measuring the absorbance of the samples at 405 nm using a microplate reader (Bio-Rad, CA, USA). Cells treated with 30 μg/mL C48/80 were used as positive controls.

2.7. Histamine release assay

LAD2 cells were cultivated to a density of 2×10^5 cells per well in a 96-well plate at 37 °C. After discarding the medium, drugs were diluted to the indicated concentrations with TM buffer and added to the wells. The plate was incubated at 37 °C for 30 min and supernatants were collected. Supernatant was applied to an HILIC column (Venusil HILIC, 2.1 mm × 150 mm, 3 μm, Agela Technologies, Tianjin, China) and isocratic elution was performed with acetonitrile-water containing 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 mL/min. Subsequent histamine analysis was performed by LC-ESI–MS/MS using an LCMS 8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan).

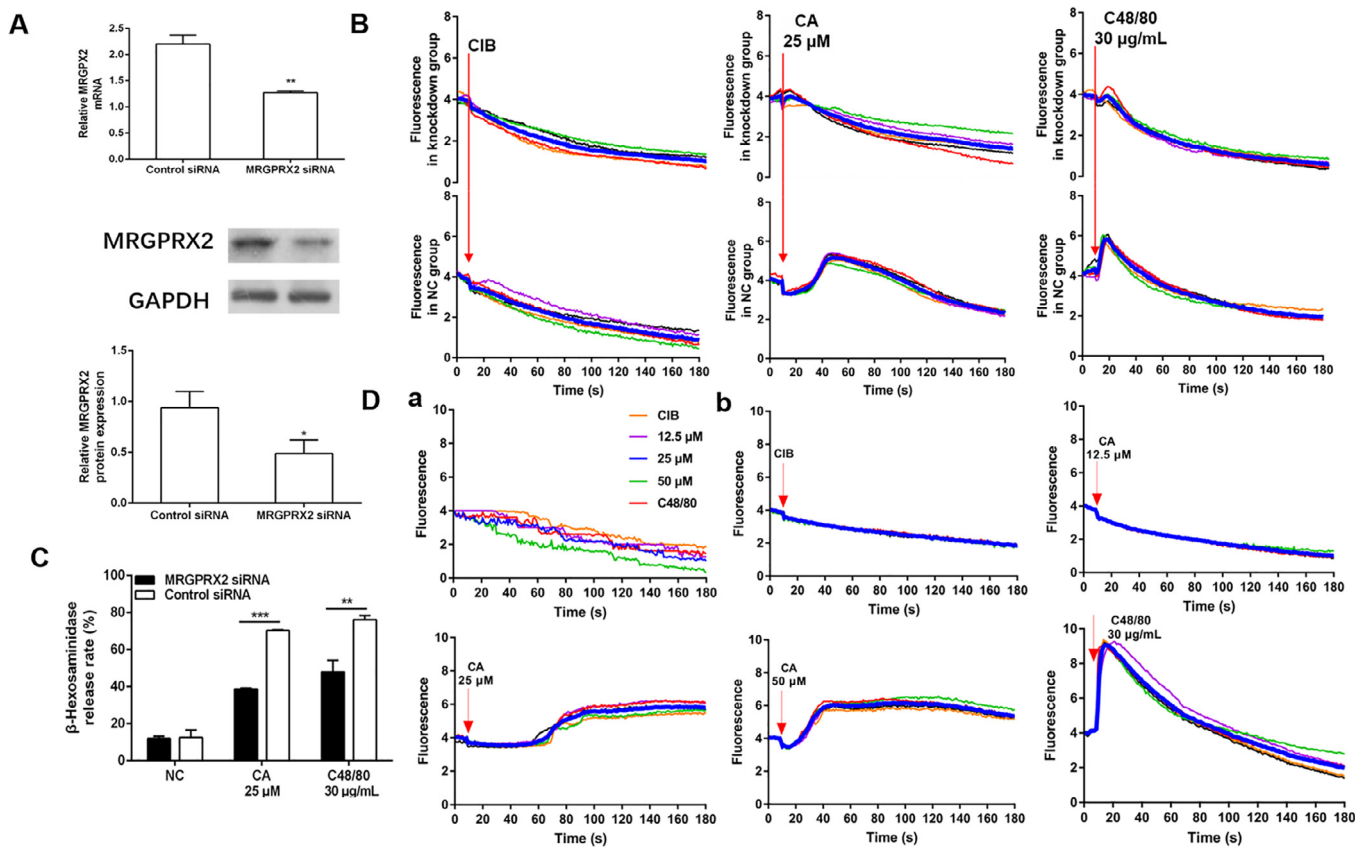

Fig. 3. MRGPRX2 plays an important role in the degranulation of MCs triggered by CA. (A) The effect of MRGPRX2-knockdown in LAD2 cells by siRNA transfection. Above, MRGPRX2 mRNA expression in LAD2 cells transfected with 1 μM negative control siRNA (NC) or MRGPRX2 siRNA using Lipofectamine 2000. Below, protein expression levels of MRGPRX2 in LAD2 cells transfected with siRNAs. (B) Representative imaging traces of Ca^{2+} concentrations in cells treated with C48/80 or 25 μM CA in transfected MRGPRX2-knockdown LAD2 cells. (C) β-hexosaminidase release in transfected MRGPRX2-knockdown LAD2 cells treated with CA for 30 min. (D) Calcium images after treatment with different doses of CA in HEK293 cells (a) and MRGPRX2-expressing HEK293 cells (b). For calcium image assays, CA was added at 10 s and each thick blue line represents the average change of fluorescence. Experiments were repeated 3 times. Data are presented as mean ± SD (n = 3). A two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < 0.05$ (*$p < 0.05$, **$p < 0\ 0.01$, ***$p < 0.001$).

2.8. Chemokine release assay

LAD2 cells were seeded into a 96-well plate at 1×10^6 cells per well and incubated for 12 h at 37 °C; drugs were added at indicated concentrations following incubation. Plate was incubated for a further 8 h at 37 °C, after which supernatants were collected. TNF-α and IL-8 detection assays were performed using ELISA Kits, according to the manufacturer's instructions.

2.9. PCA assay

Adult male C57BL/6 mice (6–8 weeks) or Mrgprb2-KO mice were anesthetized with an intraperitoneal (i.p.) injection of 3.5% chloral hydrate. Subsequently, each mouse received an intravenously (i.v.) injection of 200 μL 0.4% Evans blue (w/v, in saline). Paw thickness was measured prior to drug administration. After fifteen minutes, different doses of CA or C48/80 (30 μg/mL) were injected into the left paws; saline was injected into the right paws as a negative control. Paw thickness was measured again 15 min after drug administration. Mice were then euthanized by decapitation and paw tissues were collected, dried for 24 h at 50 °C, and weighed separately. Evans blue dye was extracted by adding 1 mL of a mixture of acetone-saline (7:3) to each tissue sample and incubating for 8 h at 37 °C. Tissues were then minced, subjected to ultrasonic disruption for 30 min, and centrifuged at 12000 × *g* for 20 min. Supernatants were transferred to 96-well plates in equal volumes, and the absorbance at 620 nm was measured using a microplate reader.

2.10. Histological analysis

Mice were anesthetized with an i.p. injection of 3.5% chloral hydrate and then 5 μL 50 μM CA, 30 μg/mL C48/80, or saline were injected into the ears. After fifteen minutes, mice were euthanized by decapitation and the tissues at the injection site were collected. Tissue samples were then washed with PBS, fixed with 4% formaldehyde for 48 h, and then subjected to hematoxylin and eosin (HE) staining. After staining, slides were dried and incubated with blocking solution (10% [v/v] normal goat serum, and 0.2% [v/v] Triton X-100 in PBS, pH 7.4) for 2 h at 25 °C. Then 1/500 FITC-avidin was added and slides were incubated for a further 45 min. Slides then washed three times with PBS, and a drop of Fluoro-mount G (Southern Biotech, AL, USA) was added. Images were captured immediately using a confocal laser scanning microscope (Nikon, Tokyo, Japan).

2.11. siRNA transfection of LAD2 cells

Specific knockdown was achieved using small interfering RNA (siRNA). The siRNA sequences were as follows: negative control siRNA forward, 5′-UUCUCCGAACGUGUCACGUTT-3′ and reverse, 5′-ACGUG ACACGUUCGGAGAATT-3′; MRGPRX2-knockdown siRNA, forward, 5′-GUACAACAGUGAAUGGAAATT-3′ and reverse, 5′-UUUCCAUUCAC UGUUGUACTT-3′. The siRNAs were delivered at a final concentration

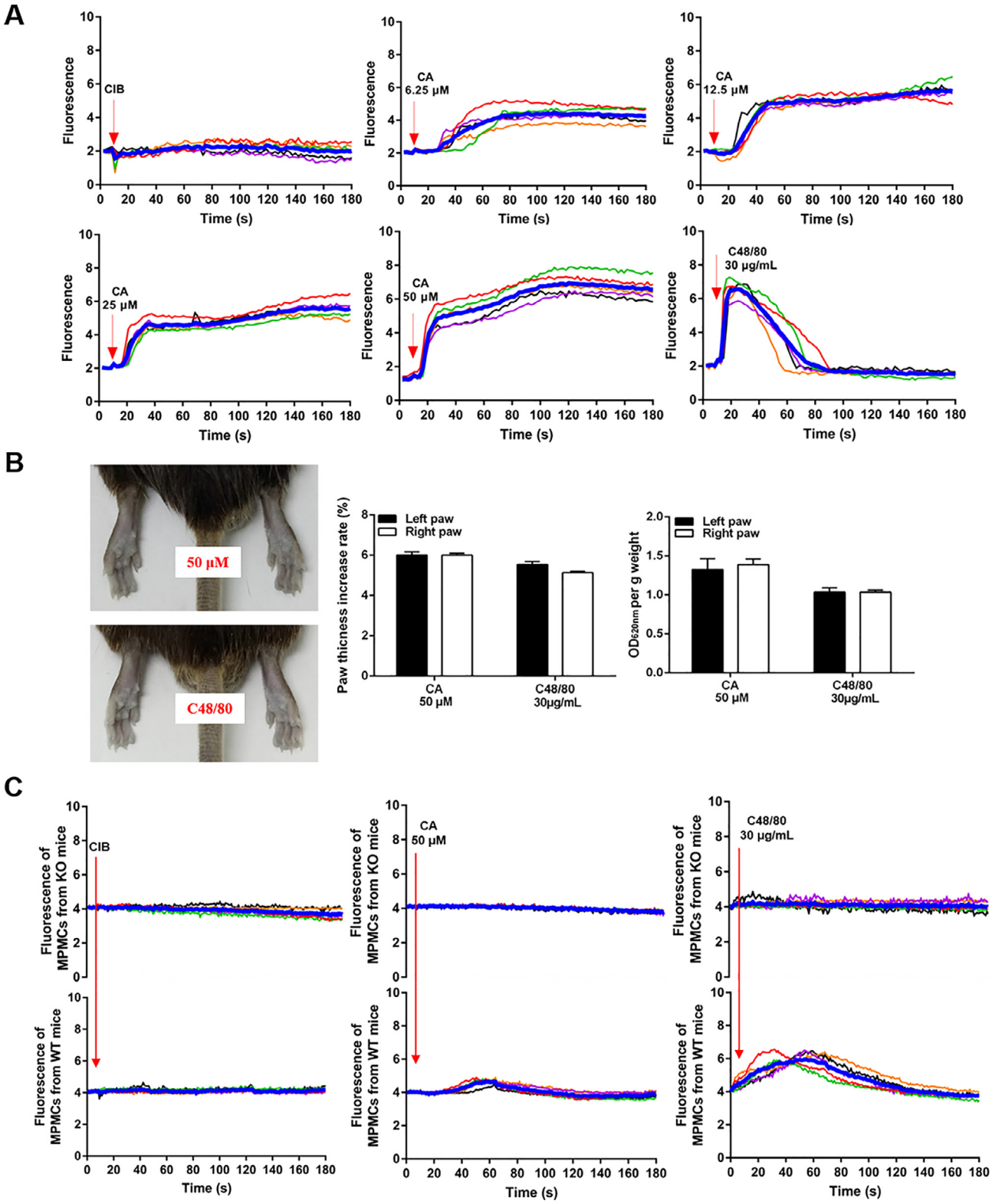

Fig. 4. CA-induced PCA was mediated by Mrgprb2 in mice. (A) CA increased intracellular calcium concentrations in Mrgprb2 cells in a dose-dependent manner. (B) Quantification of paw thickness and Evans blue extravasation in Mrgprb2-KO mice. Data are presented as mean ± SD (n = 5). (C) Calcium images after treatment with C48/80 or CA (50 μM) in MPMC isolated from Mrgprb2-KO and WT mice. For calcium image assays, CA was added at 10 s and the thick blue lines represent the average change of fluorescence. Experiments were repeated 3 times. A two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < 0.05$ (*$p < 0.05$, **$p < 0\ 0.01$, ***$p < 0.001$).

of 1 μM using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Cells were incubated for 48 h to allow for MRGPRX2 knockdown, which was then verified by RT-PCR and western blotting.

2.12. Construction of MRGPRX2-expressing HEK293 cells

HEK293 cells were seeded into a 96-well plate at 2×10^4 cells per well and incubated for 24 h at 37 °C to allow settling. Reagent A was prepared by mixing 100 μL of Opti-MEM (Gibco, Carlsbad, CA, USA) with 10 μL Lipofectamine 2000 and reagent B was prepared by mixing 125 μL of Opti-MEM with 40 μL of MRGPRX2 plasmid. Reagent A and B were then mixed in equal volumes and incubate for 5 min at room temperature. Subsequently, 10 μL of the plasmid-lipid complex was added to the cells, which were transfected by incubating for 2 d at 37 °C. Non-transfected HEK293 cells were used as a negative control to evaluate transfection quality.

2.13. MPMC purification

Adult male WT and KO mice (6–8 weeks) were euthanized by CO_2 inhalation. A total of 8 mL of ice-cold MC dissociation medium (MCDM: HBSS with 3% FBS and 10 mM HEPES, pH 7.2) was used to perform two sequential peritoneal lavages. Lavage fluid was subsequently centrifuged at $200 \times g$ for 5 min. The cell pellet from each mouse was resuspended in 2 mL cell-staining buffer (PBS with 3% heat-inactivated FBS), followed by the addition of 10 μL antibody solution and incubation for 20 min on ice. Cells were then centrifuged at $500 \times g$ for 5 min at 4 °C. MCs were washed once with 1 × BD IMag buffer (10 × BD IMag buffer diluted with PBS), resuspended in 20 μL BD IMag Streptavidin Particles Plus DM, and incubated for 30 min at 4 °C. Volume was adjusted to 2 mL with 1 × BD IMag buffer and MCs were subsequently separated using BD IMag Cell Separation Magnet.

2.14. Statistical analysis

Data are presented as mean ± standard deviation (SD) and were statistically analyzed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA). Two-tailed t-tests were used for comparisons between two groups and differences were considered significant at $p < 0.05$.

3. Results

3.1. CA induces mast cell degranulation in a dose-dependent manner

To verify whether CA-induced activation of MCs promoted degranulation, we used LAD2 cells to detect changes in intracellular Ca^{2+} concentration and secretion of β-hexosaminidase, histamine, TNF-α, and IL-8. As shown in Fig. 1A, CA increased intracellular Ca^{2+} concentration in a dose-dependent manner. As shown in Fig. 1B, different concentrations of CA (6.25, 12.5 and 25 μM) promoted the release of β-hexosaminidase into 8.70 ± 1.07%, 37.41 ± 3.25% and 50.76 ± 1.36%. Histamine concentration were 22.75 ± 0.67, 26.50 ± 3.14 and 45.68 ± 4.08 ng/mL, respectively. The secretion of TNF-α and IL-8 induced by CA were 120.97 ± 4.71, 179.52 ± 13.17, 225.77 ± 21.17 pg/mL and 18.10 ± 2.29, 21.88 ± 1.79, 30.14 ± 0.78 pg/mL. There was no significant difference in the level of inflammatory mediator release at low CA concentration compared to the negative control (NC) group. However, the inflammatory mediator release ratio increased significantly when cells were treated with CA up to 12.5 μM and 25 μM. These findings indicate that gold induced MC activation in a dose-dependent manner.

3.2. CA induces a local inflammatory reaction

In these experiments, 5 μL different concentrations of CA (12.5, 25 and 50 μM) were administered, using a microinjector, into the left paws and saline was administered into the right paws as a negative control. As seen in Fig. 2A, saline did not induce significant paw swelling, whereas the thickness of left paws increased significantly and the degree of swelling caused by CA was dose-related. In addition, we evaluated the leakage of Evans blue dye from blood vessels; although this was observed, to some extent, in the right paws, it was not due to increased vascular permeability caused by a local inflammatory reaction. Leakage from left paws always exceeded that seen in the right paws. A significant difference was observed between left paw leakage in the two groups associated with administration of up to 50 μM CA.

To further study CA-induced local anaphylaxis in WT mice, 5 μL CA (50 μM), C48/80 (30 μg/mL), or saline were injected into the ears. We then performed HE and avidin staining of ear skin sections. Prominent dilated vessels were observed after treatment with CA and C48/80 (Fig. 2B, marked with red wireframe). Furthermore, MCs were degranulated in both CA and C48/80 treatment groups (Fig. 2C). Few MCs were degranulated in the vehicle-treated control group (marked with yellow arrow), whereas the percentage of degranulated MCs (marked with red arrow) in the CA and C48/80 treatment groups were 37% and 62%, respectively. These results indicate that CA induced a local inflammatory reaction in a dose-dependent manner.

3.3. MRGPRX2 mediates CA-induced mast cell activation

To verify whether MRGPRX2 is involved in the CA-induced anaphylactoid reaction, MRGPRX2 knockdown was generated in LAD2 cells. The effects of this knockdown are shown in Fig. 3A. The changes in intracellular Ca^{2+} concentration seen with CA treatment virtually disappeared in MRGPRX2-knockdown LAD2 cells (Fig. 3B). Following treatment with CA (25 μM) or C48/80 (30 μg/mL), β-hexosaminidase release percentages in MRGPRX2-knockdown cells were 38.68 ± 0.65% and 47.94 ± 6.31%, respectively, while the same treatment in cells transfected with control siRNA resulted in 70.35 ± 0.34% and 76.19 ± 1.78%, respectively (Fig. 3C). Furthermore, we constructed MRGPRX2-expressing HEK293 cells to further determine the relationship between CA and MRGPRX2. As seen in Fig. 3D, CA could not activate HEK293 cells, but increased intracellular Ca^{2+} concentration of the MRGPRX2 high expressing cells in a dose-dependent manner. These results indicate that, CA induced degranulation via MRGPRX2 *in vitro*.

3.4. CA induces a local inflammatory reaction via Mrgprb2

Since the human MRGPRX2 gene is homologous to the mouse Mrgprb2 gene, we used Mrgprb2-expressing cells to investigate the effects of CA. Intracellular Ca^{2+} concentration increased gradually with the increase in administered CA concentration (Fig. 4A). These results indicated that CA may act on the Mrgprb2 receptor and cause a local inflammatory reaction in mice. Subsequently, we used Mrgprb2-KO mice to test this hypothesis. As shown in Fig. 4B, the paws of KO mice injected with CA did not swell and showed no obvious Evans blue dye leakage or local inflammatory reactions. These results were significantly different than those observed in WT mice. We also extracted MPMCs from WT mice and Mrgprb2-KO mice to assess the activation of MCs by CA. Fig. 4C shows that CA and C48/80 had minimal effects on MPMCs activation in Mrgprb2-KO mice compared to that seen in WT mice, which further confirmed that CA did not induce a local inflammatory reaction in Mrgprb2-KO mice. Thus, CA interacts with MRGPRX2 and triggers an MC-mediated response, resulting in a pseudo-allergic reaction.

4. Discussion

Gold is a rare metal and an important raw material for precious jewelry. However, it has been reported that gold can cause severe contact sensitivity [13]. In this study, we showed that the allergy effects of gold were mediated by the activation of MCs and demonstrated that gold can also induce MRGPRX2-associated allergies both *in vitro* and *in vivo*.

MCs are tissue-resident, multifunctional immune cells and play a critical role in host defense [14] and therefore contribute to allergic and inflammatory diseases [15,16]. MRGPRX2 is a novel G protein-coupled receptor and is expressed at high levels in human skin. LAD2 cells are skin-derived MCs that express high levels of MRGPRX2 and were therefore selected to study the effects of gold on MC activation [17].

Changes of cytosolic Ca^{2+} is an indicator of a variety of biological reactions [18]. In MCs, Ca^{2+} activates a complex cascade of signals, which leads to exocytosis of granule-associated mediators, proteases, lipid mediators, and pro-inflammatory cytokines [19]. We measured intracellular Ca^{2+} mobilization, β-hexosaminidase release, and levels of histamine, TNF-α, and IL-8. The results indicated that CA pretreatment induced LAD2 cell activation and degranulation in a dose-dependent manner; suggesting that CA, like C48/80, induces granule release in MCs.

As tissue-resident cells, MCs are distributed in host-environment interfaces, where they can readily respond to antigen stimulation [20]. We performed a skin allergy test, using skin samples containing MCs to evaluate the pseudo- allergy effects of CA. In the CA treatment group, the release of inflammatory mediators in the skin resulted in an increase in blood vessel permeability, which led to paw swelling and Evans blue dye leakage. Interestingly, when CA was injected into the left paws, the right paws of mice also showed leakage of Evans blue dye, especially in the C48/80 group. We concluded that CA and C48/80 caused a pseudo-allergic reaction and induced hemangiectasis when injected into mouse paws. This suggests that CA may enter the blood circulation and cause systemic anaphylaxis. Furthermore, we used Mrgprb2-KO mice as an *in vivo* model and MPMCs from WT and Mrgprb2-KO mice as an *in vitro* model to further confirm whether CA-induced allergy is depended on Mrgprb2. The results suggest that CA-induced skin allergic reactions involved Mrgprb2.

MRGPRX2 is an acute factor in IgE-independent allergies, therefore we used MRGPRX2-knockdown LAD2 cells and MRGPRX2-expressing HEK293 cells to further investigate the effects of CA in this context. MRGPRX2-knockdown LAD2 cells were barely activated by CA and showed a significant decrease in the release of β-hexosaminidase. Additionally, CA induced an increase in intracellular Ca^{2+} concentration in cells expressing either MRGPRX2 or Mrgprb2, in a dose-dependent manner. Thus, these results further support the idea that the pseudo-allergic effect induced by CA is dependent on MRGPRX2.

Although gold is a non-sensitizing material, it can induce severe allergies. A previous study showed that gold commonly induces IgE-mediated degranulation, but here we present another mechanism of gold sensitization. We demonstrated that gold can activate MCs directly, leading to their degranulation, and can induce a pseudo-allergic reaction. Furthermore, the occurrence of this reaction is closely related to MRGPRX2. These findings outline an additional mechanism associated with gold-induced allergic reaction and indicate that maintenance of gold jewelry is an important factor in avoiding pseudo-allergic reactions.

Acknowledgements

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Disclosure of conflict of interest

None.

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