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Baicalin induces Mrgprb2-dependent pseudo-allergy in mice

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ABSTRACT

Baicalin, a component of traditional Chinese medicine, is one of the main compounds present in *Scutellaria baicalensis* Georgi. Pseudo-allergy induced by the injection of these medicines is a frequent adverse drug reaction. Therefore, elucidation of the anaphylactoid reaction of baicalin and its underlying mechanisms are important. Mast cells are primary effectors of allergic reactions, including pseudo-allergy. Studies have shown that Mrgprx2 in human mast cells is a specific receptor that is crucial for pseudo-allergic drug reactions, Mrgprb3 is the rat ortholog of human Mrgprx2, which in mice is designated as Mrgprb2. Here, we aimed to investigate baicalin-induced pseudo-allergy and the association of Mrgprb3 and Mrgprb2 with this effect. We examined the allergenic effect of baicalin on RBL-2H3 cells and *Mrgprb3*-knockdown RBL-2H3 cells. Mrgprb2-expressing HEK293 cells and *Mrgprb2*-knockout mice were used to evaluate the role of Mrgprb2 in baicalin-induced allergy. Baicalin was found to dose-dependently induce pseudo-allergy both *in vitro* and *in vivo*. RBL-2H3 cells were activated by baicalin, whereas in *Mrgprb3*-knockout RBL-2H3 cells, baicalin showed a negligible effect on cell activation. Furthermore, baicalin activated the Mrgprb2-expressing HEK293 cells. Our data showed that baicalin did not induce allergy in *Mpgprb2*-knockout mice. We conclude that baicalin induces pseudo-allergy via Mrgprb2 in mice.

1. Introduction

Scutellaria radix, the dried root of *Scutellaria baicalensis* Georgi (family: Lamiaceae), is a traditional Chinese medicine that has been used since ancient times and is still used frequently as a well-accepted therapeutic strategy. It is used alone or in combination with other herbs in compound formulations to treat diseases such as inflammation, cardiovascular diseases, and respiratory and gastrointestinal infections [1].

Baicalin, the predominant flavonoid present in *Scutellaria radix*, shows remarkable pharmacological activities, such as the promotion of neurogenesis [2], anti-inflammation [3], anti-cancer activities [4], and several other effects [5,6]. Hence, *Scutellaria* is used as an ingredient in several traditional Chinese medicine injections (TCMIs), such as Yinzhihuang, Shuanghuanglian (SHL), and Qingkailing injections. Most of these injections are used as antipyretic analgesics, and studies have shown that baicalin reduces transient receptor potential vanilloid type-1(TRPV1) mRNA levels *in vitro* at 37 °C and 39 °C [7]. Although TCMIs possesses multiple pharmacological activities, recent studies have demonstrated an increase in the number of allergic reactions induced by these injections [8,9]. In China, approximately 400 million patients are treated with TCMIs per year. Adverse drug reactions (ADRs) have been reported with an incidence of approximately 1.51 %, representing around 67.3 % of ADRs occurring within 1 h, which are classified as immediate hypersensitivity reactions (IHRs). Among these, SHL injection has the highest incidence of ADRs. 1 % of 10000 patients who had been treated with SHL suffered from local (cutaneous or mucosal) hyperreactions, while 0.127 % of them suffered from systemic anaphylaxis [9]. It is well known that baicalin is the main active component in SHL [10]. However, the relationship between baicalin and these reactions remains unclear.

Anaphylaxis is a common adverse reaction associated with the clinical use of drugs. Mast cells (MCs) play a central role in inflammatory and allergic reactions through the release of stored or *de novo*-synthesized inflammatory mediators such as histamine, proteases, prostaglandins, leukotrienes, and cytokines. MCs are not only activated by IgE antibody via the FcεRI, but also a range of cationic substances in IgE-independent pathways or pseudo-allergy mechanisms [11,12]. These substances collectively called basic secretagogues, including inflammatory peptides and amines such as substance P (SP), mast cell

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degranulating peptide, vasoactive intestinal peptide, and compound 48/80 (C48/80). Further, a large family of G protein-coupled receptors (Mas-related genes; Mrgs) has been reported to associated with IgE-independent pathway [11]. McNeil et al. discovered that basic secretagogues directly induce pseudo-allergic reactions through Mas-related G protein–coupled receptor X2 (Mrgprx2) on MCs, which has been supported by short interfering RNA-mediated knockdown studies [13–15]. Mrgprb2 in mice is orthologous to the human G-protein-coupled receptor Mrgprx2, which is known as Mrgprb3 in rats [16,17]. Mrgprx2 is the target of small-molecule drugs associated with systemic pseudo-allergic or anaphylactoid reactions [14].

In the present study we used naïve mice and non-sensitized effector cells, which excluding IgE-mediated allergy, to study baicalin-induced pseudo-allergy in mice and determine whether this effect was related to Mrgprb2.

2. Materials and methods

2.1. Drugs and reagents

Baicalin was provided by the Chengdu Pufeide Biotech Co. Ltd. (Chengdu, China) and purified to ≥ 98 % purity. Compound 48/80 (C48/80), mass spectrometry grade formic acid, and histamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Histamine dihydrochloride (A, A, B, B-D4, 98 %) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA). Fluo-3, AM ester, and pluronic F-127 were procured from Biotium. HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, USA). Rat IL-6, IL-4 ELISA Kit were purchased from ExCell Biology, Inc. (Shanghai, China), 5-hydroxytryptamine (5-HT) ELISA kit was provided by CUSABIO (Wuhan, China).

2.2. Animals

BALB/c mice were obtained from the Experimental Animal Center of the Xi'an Jiaotong University (Xi'an, China). Mast cell-deficient W-sash c-kit mutant $Kit^{W-sh/W-sh}$ mice on the C57BL/6 background were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). *Mrgprb2* knockout mice were a kind gift from the Xinzhong Dong Laboratory of the Johns Hopkins University (MD, USA). All animals were housed in the Experimental Animal Center of Xi'an Jiaotong University at 23 − 25 ˚C with 40 % humidity and a 12 h dark and 12 h light cycle. BALB/c and C57BL/6 mice were randomly divided into the following groups: vehicle (saline), C48/80, and baicalin-treated (BALB/c mice for Active Systemic Anaphylaxis: 26.3 mg/kg, 131.5 mg/kg, and 263.1 mg/kg; C57BL/6 mice for paw injection: 6.5 mg/mL, 13 mg/mL and 26 mg/mL; 7 μL/paw).

2.3. Cell lines

Rat basophilic leukemia-2H3 (RBL-2H3) cells were provided by the cell bank of the Shanghai Biochemistry Institution. RBL-2H3 cells were cultured in DMEM (Sigma Sigma-Aldrich, St. Louis, MO, USA) with 10 % FBS (Clontech, Mountain View, CA, USA), 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen, Scotland, UK). The cells were maintained in a 5 % CO_2 incubator at 37 ˚C. Upon reaching 80 % confluence, the cells were trypsinized, re-suspended in medium, and seeded in 96- well plates or 6-cm dishes, for subsequent experiments. HEK293 cells were purchased from the ATCC. HEK293 cells expressing Mrgprb2 (henceforth called Mrgprb2-expressing HEK293 cells) were a present from Dr. Dong of the Johns Hopkins University School of Medicine.

2.4. Ethics statement

This study was performed in strict accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health, USA. The experimental protocols for using BALB/c mice were also approved by the Animal Ethics Committee of the Xi'an Jiaotong University (permit number: XJTU 2011-0045).

2.5. SiRNA transfection of RBL-2H3 cells

Specific knockdown was achieved using either small interfering RNAs (siRNAs) targeting Mrgprb3 or a negative control siRNA. A smart pool of double-stranded siRNAs targeting Mrgprb3 as well as non-specific siRNAs was obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences were as follows: Negative Control siRNA, forward, 50-UUCUCCGAACGUGUCAC GUTT-30, and reverse, 50-ACGUGACACGUUCGGAGAATT-30; MRGPRX2 knockdown siRNA, forward, 50-GGAUCUAUUGGUUCCUCUUTT-30, and reverse, 50-AAGAGGAACCAAUAGAUCCTT-30. 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 Mrgprb3 knockdown.

2.6. Degranulation assay

The RBL-2H3 cells display properties of mucosal-type MCs, and they have been commonly and successfully used in *in vitro* studies on degranulation, with the release and generation of several inflammatory mediators [18–20]. RBL-2H3 or *Mrgprb3* knockout (RBL-$2H3^{MUT}$) cells in logarithmic growth phase were seeded in a 96-well plate at 2 × 10^4 cells/well and incubated overnight at 37 ˚C in the presence of 5 % CO_2. The culture medium was removed, and different doses of baicalin (25 μM, 50 μM, 100 μM, dissolved in Tris-Magnesium Sulphate buffer) or C48/80 (30 μg/mL) were added into each well and incubated for 30 min at 37 ˚C in the presence of 5 % CO_2. The reactions were terminated after 30 min by placing the plate on ice for 10 min, and the supernatant was collected and centrifuged at 100*g* for 10 min at 4 ˚C to remove cells and debris. The remaining cells of vehicle group in the plate were lysed by treatment with assay buffer containing 1 % (v/v) Triton X-100 prior to incubation with β-hexosamine. Hexosaminidase activity was determined by incubating the supernatant and cell lysate with 1 mM β-hexosamine for 90 min at 37 ˚C. The reaction was terminated by adding 0.1 μM Na_2CO_3/$NaHCO_3$ and the absorbance was measured at 405 nm. The β- hexosaminidase released was calculated using the following formula: β-hexosaminidase release rate (%) = [absorbance of sample cell supernatant /vehicle cell supernatant + absorbance of vehicle cell lysate] × 100.

2.7. Inflammatory factors and 5-HT detection

RBL-2H3 cells were cultured in a 96 well plate for 24 h at 37 ˚C, and different doses of baicalin (25 μM-100 μM) or C48/80 (30 μg/mL) were added into each well and incubated for 30 min at 37 ˚C in the presence of 5 % CO_2. The supernatant of each well was collected, IL-6, IL-4 and 5-HT were measured with ELISA followed the manufacturers' instructions.

2.8. Calcium imaging

RBL-2H3 cell**s,** RBL-$2H3^{MUT}$ cells, Mrgprb2-expressing HEK293 cells and HEK 293 cells were plated in a 96-well plate (1 × 10^4 cells/well) and incubated for 24 h at 37 ˚C, respectively. Then, the cells were washed twice with CIB (calcium imaging buffer; 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, pH adjusted to 7.4 with NaOH and stored

at 4 ˚C), and loaded with the incubation buffer (3.5 μM Fluo-3 AM, 0.1 % (w/v) F-127, diluted with CIB) at 37 ˚C for 30 min, following by washing twice with CIB, and used immediately for imaging. Ca^{2+}-flux in the cells was monitored by fluorescent microscopy on an inverse microscope (Nikon, Ti-U, Japan; excitation wave length: 480 nm) by taking 200 pictures at a frequency of 1 /s)

2.9. Histamine release

Histamine levels were evaluated using liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry (LC-ESI-MS/MS) on an LCMS 8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) and a HILIC column (Venusil HILIC, 2.1 mm × 150 mm, 3 μm; Agela Technologies, Tianjin, China). The analysis was conducted using isocratic elution with a mixed solute ion of acetonitrile–water with 0.1 % formic acid and 20 mM ammonium formate (77:23, v/v); the flow rate was 0.3 mL/min.

2.10. Analysis of active general anaphylaxis in mice

Analysis of active general anaphylaxis was performed according to our previous report [21]. In brief, adult male mice (20 − 25 g) were anaesthetized with an intraperitoneal (i.p.) injection of 0.3 g/kg chloral hydrate and then administered intravenously with C48/80 (2.5 mg/kg), baicalin (26.3, 131.5 or 263.2 mg/kg) or the vehicle through the caudal vein, combined with 0.4 % Evans Blue (n = 6). The animals were euthanized 30 min after this challenge, and their ears were removed and processed to measure the dye extravasation after the dye had been extracted overnight from the ear with 800 μL of a mixture of acetone-saline (7:3) at 65 ˚C. The absorbance was measured at 620 nm.

2.11. Paw swelling and extravasation

Adult male mice (20 − 25 g) were anaesthetized with an intraperitoneal injection of 0.3 g/kg chloral hydrate. Each mouse was injected intravenously (i.v.) with 0.2 mL 0.15 % Evans Blue in PBS. The thickness of the paw before injection was measured using a vernier calliper. After 5 min, 7 μL baicalin (6.5 mg/mL, 13.0 mg/mL, and 26.0 mg/mL) or C48/80 (10 μg/mL) was injected into the left paw with a microinjector. An equal volume of PBS was injected into the right paw as a negative control, and paw thickness was measured 5 min after the injection. The mice were sacrificed, paws were separated, cut into small pieces and collected in 2 mL epoxy tubes, classified, dried at 50 ˚C, and weighed. Evans Blue was subsequently extracted by adding 1 mL mixture of acetone-saline (7:3) at 37 ˚C for 12 h. The supernatant was distributed into a 96-well cell culture plate (200 μL/well) and the OD was measured at 620 nm with a spectrophotometer.

2.12. Assay to determine changes in body temperature

All mice were housed in the procedure area the day before the injection. Both the wild-type (WT) and mutant mice (male, 20 − 25 g) were randomly divided into four groups (4 mice in each group). Anesthesia was induced by i.p. injection of 0.3 g/kg chloral hydrate. The tail veins were dilated by repeated wiping of the tail with a tissue soaked in 100 % ethanol, followed by injection of baicalin (260 mg/kg). The body temperature was recorded using a biological function experimental system (Taimeng, Chengdu, China) in which a probe was inserted into the anus of the mouse for 30 min.

2.13. Histological analysis

Mice left paws were injected with 5 μL of baicalin or C48/80, vehicle was used as a negative control. Fifteen minutes later, the injection site of the skin in paw was excised, washed with PBS and fixed with 4 % formaldehyde for 48 h and subjected to H&E staining or Avidin staining. Avidin was shown to selectively bind to heparin glycosaminoglycan and to bind highly preferentially to MCs granules rather than to other cellular structure. In human subjects and rodents avidin conjugated to fluorochrome was shown to selectively stain MCs in fixed preparations/tissues. For Avidin staining [22], slides were pre-incubated with the blocking solution (10 % normal goat serum (v/v), 0.2 % Triton X-100 (v/v) in PBS, pH 7.4) for 2 h at 25 ˚C, followed by incubation with 1/500 FITC-avidin for 45 min. Sections were washed three times with PBS, and a drop of Fluor-mount G (Southern Biotech, AL. U.S.A) was added. Images were captured immediately using a fluorescence microscope (Nikon, Tokyo, Japan). Non-degranulated MCs have a clear outline and regular shape, containing characteristic intracellular granules. Degranulated MCs were identified by the appearance of large fluorescent green granules outside of MCs with a lower content of condensed intracellular granules [23]. The percentage of degranulation ratio (%) = degranulated MC number/ (degranulated MC number + non-degranulated MC number) * 100 %.

2.14. Statistical analysis

The data are presented as the mean ± standard deviation (SD) and were statistically analyzed using analysis of variance (ANOVA). Two-tailed tests were used for two-group comparisons and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Baicalin induces an inflammatory response in mice

To investigate baicalin-induced pseudo-anaphylactic reaction *in vivo*, different doses of baicalin and Evans Blue (4 %) were injected intravenously into the caudal vein of BALB/c mice. C48/80-treated mice were used as positive control. Baicalin treatment increased dye diffusion by 50 %, suggesting that baicalin increased ear vessel permeability (Fig. 1a). We also assessed the effects of baicalin using a local anaphylaxis test. Evans Blue was injected intravenously and after 5 min, baicalin was injected subcutaneously into the left paw of the mice. Baicalin induced extensive extravasation and swelling in C57BL/6 WT mice in a dose-dependent manner (Fig. 1b). Subsequently, we observed remarkable hemangiectasis caused by baicalin (Fig. 1c). Together, these data indicated that baicalin could induce pseudo-allergic reactions in mice.

3.2. Baicalin induces MC-dependent pseudo-allergic reactions in mice

Local application of baicalin has been proved to induce passive cutaneous anaphylaxis in WT mice (Fig. 1b). We then labeled the MCs in skin at the application site using avidin and found a significant increase in the percentage of degranulated MCs upon baicalin treatment (Fig. 2a), which suggested that baicalin activated MCs *in vivo* to cause degranulation.

Mast cell-deficient W-sash c-kit mutant $Kit^{W-sh/W-sh}$ mice carrying spontaneous loss-of-function mutations at both alleles of the dominant white spotting (W) locus (ie, c-kit), exhibit a marked reduction in c-kit tyrosine kinase-dependent signaling, resulting in disrupted normal mast cell development and survival and therefore mast cell function [24]. We used this model to further investigate whether baicalin induced allergy is related to MCs. The results showed that neither C48/80 nor baicalin induced local anaphylaxis (Fig. 2b left part). Both the degree of swelling (Fig. 2b) and Evans Blue dye (Fig. 2b) exudation were similar in the treated mice and the negative control. These data indicated that baicalin leads to a pseudo-allergic reaction via MCs.

3.3. Baicalin induces effector cell activation

RBL-2H3 cells were treated with baicalin (25 μM, 50 μM, and 100

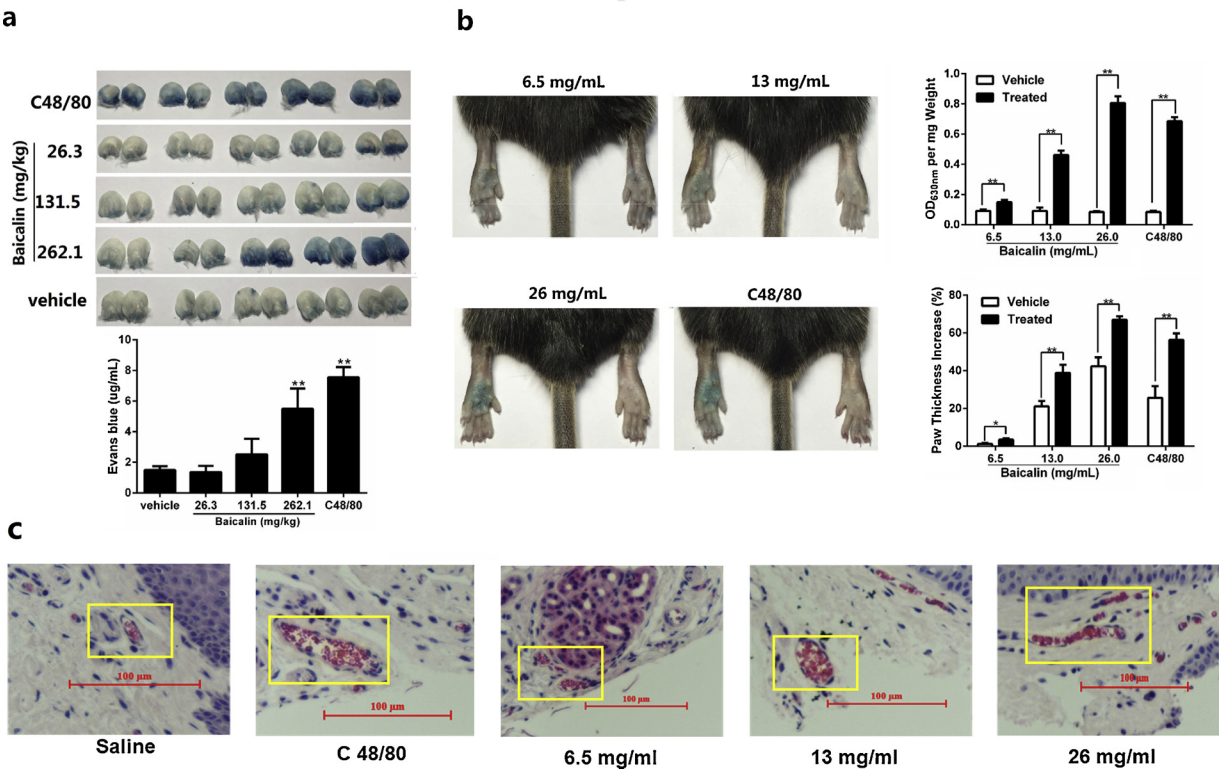

Fig. 1. Baicalin induces pseudo-allergic reactions in mice. a. Evans Blue leakage into the ears in a dose-dependent manner 15 min after administration of baicalin. b. Evans Blue leakage into the paw and paw thickness increased 15 min after administration compared to those in the vehicle treatment. c. Baicalin induced hemangiectasis as demonstrated by H&E staining of skin tissue sections. (mean ± SD, n = 8, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

μM) and C48/80. The response of RBL-2H3 cells to baicalin was determined by cellular Ca^{2+} imaging, β-hexosaminidase and histamine release assays. Intracellular calcium levels ($[Ca^{2+}]_i$) in RBL-2H3 cells increased significantly 10 s after baicalin stimulation (Fig. 3a). Further, histamine, β-hexosaminidase and 5-HT releases were markedly enhanced by highest concentration of baicalin (100 μM, Fig. 3b). The release of histamine increased slightly with 25 μM and 50 μM baicalin. The release of the inflammation factors, IL-4 and IL-6, were also induced by baicalin dose-dependently (Fig. 3c).

3.4. Baicalin-induced effector cell activation depends on the presence of Mrgprb3

RBL-2H3^{MUT} cells were treated with 25 μM, 50 μM, 100 μM baicalin or C48/80 respectively. Baicalin could not activate RBL-2H3^{MUT} cells (Fig. 4a). Further, we compared baicalin-induced degranulation in the presence and absence of Mrgprb3 at the same concentrations. Both β-hexosaminidase and histamine released by the RBL-2H3^{MUT} cells were lower than those released by NC RBL-2H3 cells (Fig. 4b). Therefore, we

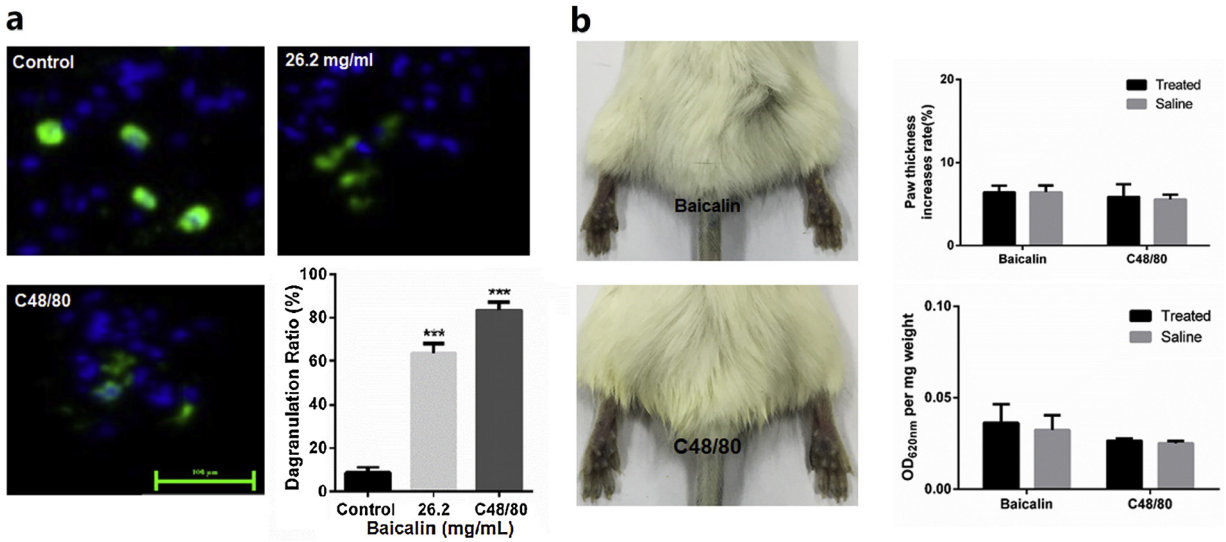

Fig. 2. Baicalin-induced pseudo-allergy in mice depends on mast cells. a. Avidin staining of the paw skin after treatment with vehicle, baicalin, or C48/80. $**P < 0.01$ vs. control. b. Representative images of Evans Blue stained extravasation 15 min after intraplantar injection of 7 μL of baicalin (left paw), C48/80 (left paw), or saline (right paw). (mean ± SD, n = 8, $***p < 0.001$).

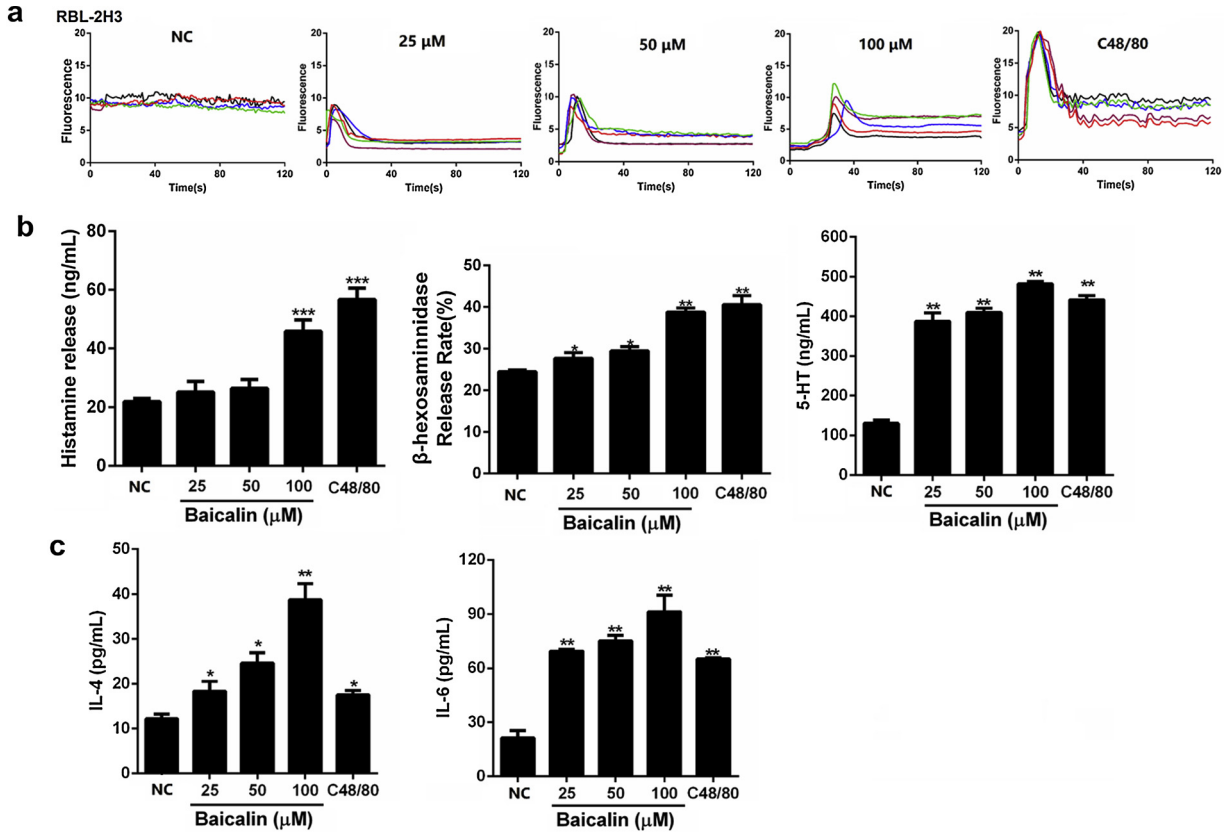

Fig. 3. Baicalin induces an anaphylactoid reaction in RBL-2H3 cells, CIB as negative control (NC) and C48/80 as a positive control. a. RBL-2H3 cells showing changes in calcium induced by baicalin in different doses. b. Baicalin induced RBL-2H3 cells degranulation dose dependently. c. Baicalin induced inflammatory factors release from RBL-2H3 cells. (mean ± SD, n = 4). ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. NC group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. each dose group respectively).

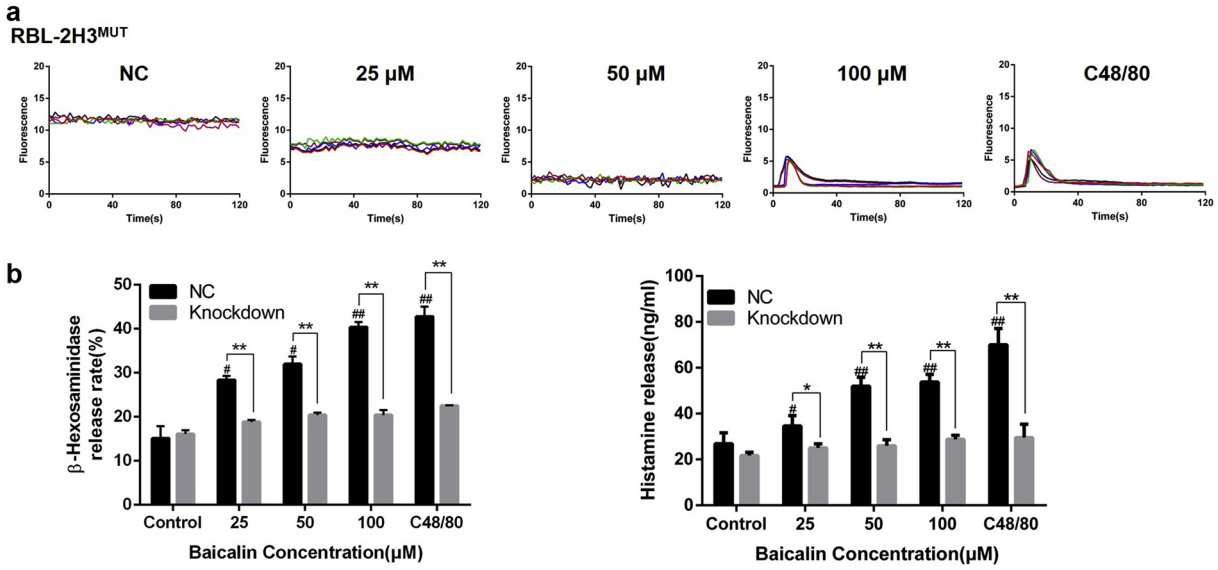

Fig. 4. Mrgprb3 is essential for RBL-2H3 cells activation by baicalin. a. Intracellular Ca^{2+} levels in RBL-2H3^{MUT} cells after stimulation with different doses of baicalin. b. Baicalin-induced RBL-2H3 cells degranulation is dose-dependent and involves Mrgpb3. (mean ± SD, n = 4). ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. NC group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. each dose group respectively).

concluded that baicalin-induced RBL-2H3 cell activation is related to *Mrgprb3*.

3.5. Baicalin-induced pseudo-allergy in mice depends on the presence of Mrgprb2

We evaluated the different effects of baicalin in the presence and absence of Mrgprb2 *in vitro*. Baicalin activated Mrgprb2-expressing HEK293 cells in a dose-dependent manner (Fig. 5a). However, HEK293

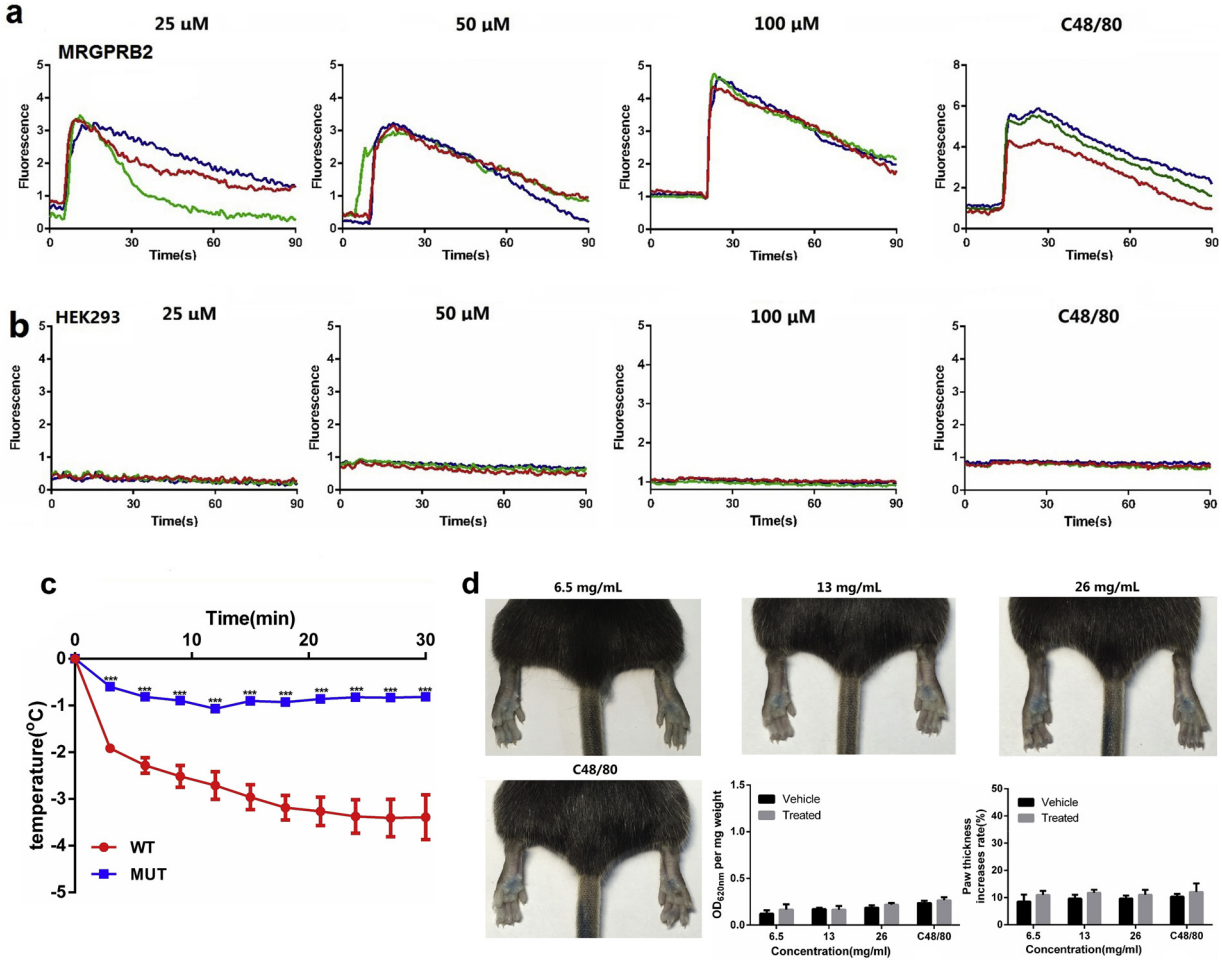

Fig. 5. Baicalin-induced pseudo-allergy in mice depends on the presence of Mrgprb2. Changes in Ca^{2+} levels in HEK293 cells expressing Mrgprb2 (a) and HEK293 control cells (b) after stimulation with different doses of baicalin. c. Lasting drop of body temperature in C57/BL6 cells 3 min after injection of baicalin (red line), but no changes in Mrgprb2 ko mice (blue line). d. The latter also showed no changes in vessel permeabilization or paw swelling 15 min after administration of baicalin plus Evans Blue. (mean ± SD, n = 6, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

control cells had no response to baicalin or C48/80.

We then used the *Mrgprb2*^{MUT} C57BL/6 mice to further investigate our observations regarding the association of baicalin with Mrgprb2. Baicalin (260 mg/kg) induced a rapid and irreversible drop in body temperature of WT mice, with a temperature reduction of 4 °C after 30 min of baicalin treatment. In contrast, *Mrgprb2*^{MUT} mice showed a smaller reduction in temperature that recovered rapidly (Fig. 5c). Baicalin (26 mg/mL) induced extensive swelling in WT mice, but not in *Mrgprb2*^{MUT} mice (Figs. 1b and 5 d). No significant differences in Evans Blue leakage or paw swelling were observed between the baicalin groups and the vehicle group (Fig. 5d). These observations demonstrated that baicalin induces anaphylactic reactions via Mrgprb2 in mice.

4. Discussion

Baicalin is the active compound in various types of TCMIs, although there is a low incidence of TCMI-induced ADRs, the life-threatening outcomes cannot be ignored. In 2018, Gao et al. reported that Shuang-Huang-Lian injection (SHL) induces an IgE-independent immediate hypersensitivity reaction [9], but the specific compound in SHL that caused the ADR remains unclear. In our previous study, we used an RBL-2H3/CMC online LC/MS system to screen for allergenic components in SHL and identify baicalin as a potential allergenic component [25]. Hence, we focused on the allergenic effects of baicalin, which may be one of the factors responsible for the adverse reactions induced by *Scutellaria baicalensis* Georgi.

In the present study, we investigated the mechanisms of baicalin-induced pseudo-allergy. Results obtained both BALB/c and C57/BL6 mouse models showed that baicalin induced mice pseudo-anaphylactic reactions when the intravenous dose reached 263.1 mg/kg (Fig. 3a), which would be approximately 29 mg/kg when converted to a human adult dose. We infered that it would be not safe to adminstrater baicalin at doses above 29 mg/kg. Our results indicated that baicalin induced local anaphylaxis in C57BL/6 WT mice, but not in mast cell–deficient Kit$^{W-sh/W-sh}$ mice. Thus, baicalin caused anaphylactoid reactions via MCs, and Mrgprb2 may play a facilitating role in both MC degranulation and pseudo-anaphylaxis in mice.

There are two main pathways for MC activation, namely, the IgE-dependent and the IgE-independent pathways [14]. Measurement of serum IgE concentration in mice after baicalin treatment revealed that baicalin did not induce IgE generation (supplement Figure). However, signs of inflammation were obvious in these mice.

MCs are primary effectors in inflammatory and allergic reactions. Cytosolic Ca^{2+} is a universal and highly versatile signal [26]. In MCs, Ca^{2+} activates a complex cascade of signals, which leads to the degranulation of pre-stored mediators, *de novo*-synthesized lipid mediators, pro-inflammatory cytokines, and chemokines [27,28]. The results of *in vitro* experiments suggested that baicalin induced intracellular Ca^{2+} in RBL-2H3 cells, which may cause degranulation and the release of inflammatory factors. Cell degranulation did not occur after baicalin or C48/80 treatment in the *Mrgprb3*-knockdown cells, indicating that

cell degranulation depends on Ca^{2+}, an important second messenger in the G-protein signaling pathway. These results indicated that baicalin-induced RBL-2H3 activation depends on *Mrgprb3*.

Above is focused on *Mrgprb3* which is existed in rat. We performed further studies to assess the effects of baicalin in mice, which express Mrgprb2. We evaluated the effect of Baicalin on Mrgprb2-expressing HEK293 cells. Baicalin was also found to activate Mrgprb2-expressing HEK293 cells (Fig. 5). The baicalin-induced activation of HEK293 cells depended on the presence of Mrgprb2. Body temperature decline is an obvious phenomenon when anaphylaxis occurs [29]. Our data showed that baicalin induced a decrease in the body temperature of mice, which depends on the presence of Mrgprb2 (Fig. 5c). The extent of Evans Blue dye leakage and paw swelling showed no significant differences between baicalin- and vehicle-treated mice. The results of the *in vivo* studies showed that almost no allergic symptoms were elicited by either C48/80 or baicalin in the *Mrgprb2*^{MUT} mice. Therefore, we concluded that the sensitization by baicalin is mediated by Mrgprb2 in mice. Mitogen-activated protein kinase (MAPK) signaling pathway consist of ERK, P38 and JNK. It was previously demonstrated that the ERK MAPK signal pathway was involved in Mrgprx2-mediated MCs activation, including the secretion and production of histamine and TNF-α [30]. Some reports also demonstrated that the phosphorylation of P38 was induced by Mrgprx2 agonists [31]. Baicalin is reported to upregulate the ERK/P38 MAPK pathway in human breast cancer cells [32]. Therefore, we speculated that baicalin-induced RBL-2H3 cell activation is related to ERK/P38 MAPK pathway which need further study.

These findings revealed the mechanism of the baicalin-induced development of allergy and provided insights regarding the clinical use of baicalin.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2020.07.006.

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