

Resveratrol inhibits MRGPRX2-mediated mast cell activation via Nrf2 pathway

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ARTICLE INFO

Keywords:

Nrf2
Mast cell
Resveratrol
MRGPRX2

ABSTRACT

Mast cells (MCs) are crucial effectors in inflammation and allergic reactions. The Mas-related G-protein-coupled receptor X2 (MRGPRX2) was the MC-specific receptor and play a key role in IgE-independent allergic reactions. The activation of the Nuclear factor erythroid derived 2-related factor 2 (Nrf2) is involved in IgE-mediated MC degranulation. Resveratrol (Res) is a polyphenolic compound in red wine and has been reported to exert a variety of pharmacological effects. In the current study, we investigated the effect of Res in regulating MRGPRX2-mediated MC activation and its underlying mechanism. We demonstrated that Res reduced compound 48/80 (C48/80)-induced calcium flux in MCs and inhibited MCs degranulation *in vitro*. Res also suppressed C48/80-induced hind paw extravasation, active systemic anaphylaxis, and MCs degranulation in mouse models of pseudo-allergy *in vivo*. Furthermore, PCR and immunohistochemistry assay suggest that Res up-regulates Nrf2 expression and Nrf2 inhibitor attenuates the protective effects of Res. In conclusion, Res exerts an inhibitory effect on MRGPRX2-mediated MCs activation by targeting Nrf2 pathway and may present a promising new therapeutic agent for the treatment of MRGPRX2-dependent anaphylactoid reactions.

1. Background

Anaphylaxis is a severe systemic hypersensitivity reaction which is associated with skin, airways, conjunctiva and gastrointestinal tract change [1]. Mast cells (MCs) are viewed as major effector cells in allergies and anaphylaxis, which are widely distributed throughout vascularized tissues, particularly near surfaces exposed to the external environment. The activation of MCs leads to degranulation and secretion of several biologically active products which participate in inflammation and allergic reactions, such as histamine and tryptase [2]. IgE is thought to have a central role in MCs activation by cross-linking of the high affinity FcεRI [3]. In addition to FcεRI, MCs also express Mas-related G protein-coupled receptor X2 (MRGPRX2) for ligands as diverse as neuropeptides (substance P, SP), morphine and compound 48/80 (C48/80) [4], which is related to pseudo-allergy or IgE-independent allergy. MRGPRX2 were determined to be targets of many commonly used small-molecule drugs associated with anaphylactoid reactions, such as fluoroquinolones, antimicrobials and neuromuscular blocking drugs [5–7]. The signs and symptoms of allergy

produced by these drugs ranging from bronchoconstriction, hypotension, itching, cramping, diarrhea, or cutaneous wheal and flare responses, which are the same as those produced by C48/80 [8]. Furthermore, MRGPRX2 has also been reported to participate in asthma and Chronic Urticaria [8,9].

Nuclear factor erythroid derived 2-related factor 2 (Nrf2) is one of the most important regulators in cellular stress response against inflammation and oxidative stress [10]. Recently, it was found to modulate MRGPRX2-mediated human MC degranulation in the hair follicle [11]. The Nrf2 target gene, heme-oxygenase 1 (HO-1), could inhibit IgE-mediated degranulation of RBL-2H3 cells through bilirubin [12–14]. Further, Nrf2/HO-1 pathway has been reported to be involved in IgE-dependent allergy [15]. Therefore, Nrf2 becomes an attractive target for the treatment of MC-mediated allergic disorders.

Resveratrol (*trans*-3, 5, 4'-trihydroxystilbene, Res) (Fig. 1A), a polyphenolic compound especially abundant in grapes and red wine, has been reported to exert a variety of pharmacological effects, including antioxidative [16], anticancer [17], and neuroprotective properties [18]. The safety, and pharmacokinetics of Res have been documented in

Abbreviations: MRGPRX2, Mas-related G protein-coupled receptor X2; Nrf2, nuclear factor (erythroid-derived 2)-like-2; SFN, sulforaphane; MC, mast cell; HO-1, heme-oxygenase 1; PGD2, prostaglandin D2; Res, resveratrol; C48/80, compound 48/80.

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<https://doi.org/10.1016/j.intimp.2021.107426>

Received 17 October 2020; Received in revised form 14 January 2021; Accepted 19 January 2021

Available online 4 February 2021

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over 244 clinical trials. Res was reported to be safe at doses up to 5 g/d when used in therapy [19]. Several studies have confirmed that Res could inhibit the IgE-mediated MC activation [20,21] and exhibited anti-asthmatic effects in a murine model of asthma and suppressed PMA plus A23187-induced expression of pro-inflammatory mediators by decreasing NF- κ B and MAPK pathway [22,23]. Furthermore, Polydatin, a natural precursor of Res, could inhibit the IgE-mediated MC activation [15]. Although it was earlier reported that Res inhibited C48/80-induced MC activation [24,25], *in vivo* effects and mechanisms demonstration of Res on anti-pseudo-allergy have not been elucidated. Therefore, we aim to investigate the preventive effect of Res on MC activation and its underlying mechanisms.

2. Materials and methods

2.1. Drugs and reagents

Res and Sulforaphane (SFN) were supplied by Chengdu Pufei De Biotech Co. Ltd. (Chengdu, Sichuan, China) and the purity is $\geq 98\%$ based on HPLC analysis. ML385 was from MedChemExpress (Shanghai, China). C48/80, p-nitrophenyl N-acetyl- β -D-glucosamide, Triton X-100, Histamine, formic acid (mass spectrometry grade) and Mass spectrometry grade formic acid were from Sigma-Aldrich (St. Louis, USA). Fluo-3, AM ester, and Pluronic F-127 were from Biotium (California, USA). StemPro™-34 medium, L-glutamine, human stem cell factor, penicillin-streptomycin were provided by Cell Signal Technology (MA, USA), Cell Counting Kit was from Abbkine Inc. (California, USA). Histamine•2HCl (A, A, B, B-D4, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA), HPLC-grade methanol and acetonitrile were from Thermo Fisher Scientific (Pittsburgh, USA). All ELISA Kits were from ExCell Bio CO., LTD. (Beijing, China) Tyrod's solution was prepared fresh on the day of use (6.954 g/L NaCl, 0.353 g/L KCl, 0.282 g/L CaCl₂, 0.143 g/L MgSO₄, 0.162 g/L KH₂PO₄, 2.383 g/L HEPES, 0.991 g/L glucose, and 1 g/L BSA, pH = 7). Stop buffer contained a mixture of 0.1 M sodium carbonate and sodium bicarbonate (pH = 11). All aqueous solutions were prepared using ultrapure water produced by MK-459 Millipore Milli-Q Plus ultra-pure water system. Trizol extraction kit

was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Primers and probes for NADP(H) dehydrogenase-quinone 1 (NQO1), HO-1, TNF- α , MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Takara (Dalian, China).

2.2. Ethical considerations

This study was conducted in strict accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental protocols for the mouse experiments were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2019-711).

2.3. Mice

C57BL/6 mice (8-week-old) were from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Young adult male mice aged 6–8 weeks were used in all the experiments in this study. All experiments involving equal treatments of animals were conducted by experimenter blind to the experimental conditions.

2.4. Cell lines

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by Prof. A. Kirshenbaum and Prof. D. Metcalfe (NIH, USA). Cells were maintained in StemPro™-34 medium supplemented with 1% StemPro™ nutrient supplement, 1:100 penicillin-streptomycin, 2 mM L-glutamine, and 100 ng/mL human stem cell factor in 5% CO₂ at 37 °C. The culture medium was replaced per week and cells maintained at a density of 2×10^6 cells/mL.

2.5. Preparation of peritoneal mast cell (MPMCs)

Mice were euthanized via CO₂ inhalation. A total of 12 mL of ice-cold mast cell dissociation media (MCDM; HBSS with 3% FBS and 10 mM HEPES, pH 7.2) was used to make two to three sequential peritoneal

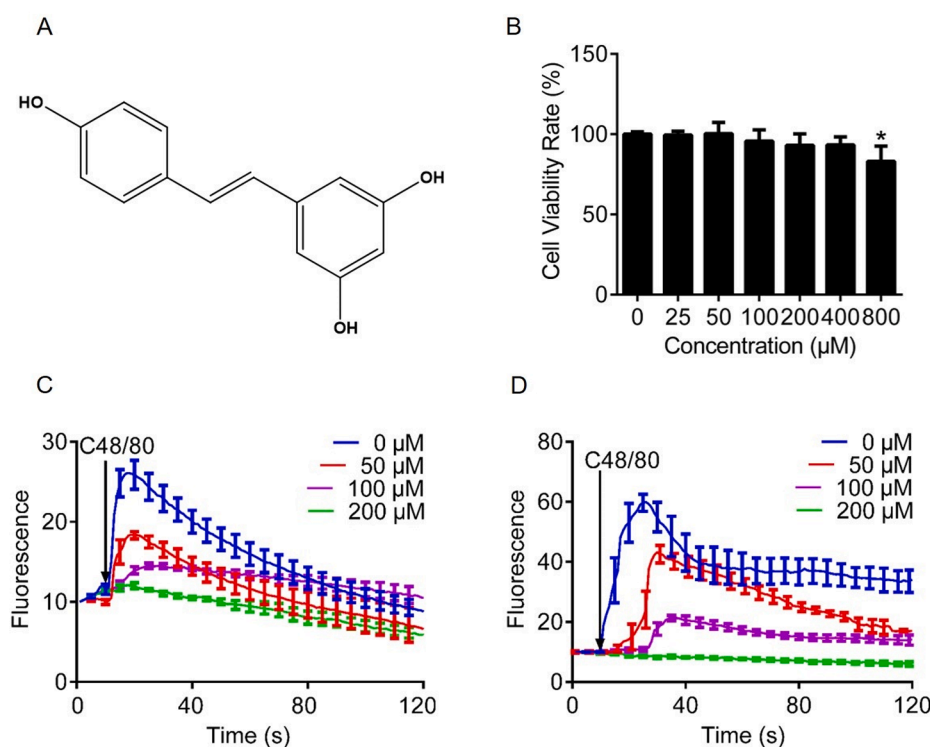


Fig. 1. Res reduces C48/80-induced calcium (Ca²⁺) in flux in mast cells. A. Chemical structure of Resveratrol (Res). B. Viability of LAD2 cells treated with Res for 24 h. C. Representative Ca²⁺ imaging traces in LAD2 cells in response to 0, 50, 100 or 200 μM Res and 30 μg/mL C48/80. D. Representative Ca²⁺ imaging traces in MPMC cells in response to 0, 50, 100 or 200 μM Res and 30 μg/mL C48/80. Data are presented as mean ± S.D. and are representative of three independent experiments with three samples per experiment (50 cells were counted per condition). ANOVA was used to determine significance in statistical comparisons (**P* < .05, compared with 0 group).

lavages, which were centrifuged at 200g and 4 °C for 5 min. The cells from each mouse were resuspended in 2 mL MCDM, layered them over 4 mL of an 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 500g and 4 °C for 20 min. The purity of the pelleted mast cells was >95% as assayed by morphology and toluidine blue staining. Mast cells were resuspended at concentrations of 5×10^5 cells/mL in DMEM supplemented with 10% FBS, 100 U of penicillin–streptomycin and 25 ng/mL recombinant mouse stem cell factor (mSCF).

2.6. Cytotoxicity assays

Cell viability was determined using Cell Counting Kit assays. LAD2 cells (5×10^3 cells/well in a 96-well plate) were treated with 90 µL of Res at different concentrations (0, 6.25, 12.5, 25, 50, 100, 200, 400, 800 µM) for 24 h. Next, 10 µL of Cell Counting Kit solution was added to each well followed by incubation for 2 h. Further, the relative cell viability was assessed by detection of absorbance at 450 nm using a microplate reader (Bio-Rad, Carlsbad, CA, USA).

2.7. β -hexosaminidase assay

LAD2 cells (1×10^4 /well in a 96-well plate) were incubated with different concentrations of Res (50, 100, 200 µM) for 1 h, followed with C48/80 (30 µg/mL) for 30 min. Cells were lysed with 0.1% Triton X-100 in tyrodés solution to analyze the total β -hexosaminidase content. The β -hexosaminidase released into the supernatants and in cell lysates was quantified by 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 stopped by adding stop buffer. The absorbance was measured at 405 nm using a microplate reader and the percentage of β -hexosaminidase release was calculated as follows: absorbance of culture supernatant at 405 nm $\times$ 100/absorbance of total cell lysate supernatant at 405 nm.

2.8. Histamine release assay

LAD2 cells (1×10^4 /well in a 96-well plate) were incubated with different concentrations of Res (50, 100, 200 µM) for 1 h, followed with C48/80 (30 µg/mL) for 30 min. The supernatants and cell lysates were collected. Histamine release assay was carried out using a LC-ESI-MS/MS method which is reported previously [26].

2.9. Chemokine release assay

LAD2 cells (1×10^4 /well in a 96-well plate) were incubated with different concentrations of Res (50, 100, 200 µM) for 1 h, followed with C48/80 (30 µg/mL) for 6 h at 37 °C. Human TNF- α , MCP-1, LTB4 and IL-6 concentrations in the supernatant were detected with ELISA kits according to provided instructions.

2.10. Intracellular Ca^{2+} mobilization assay

LAD2 cells were plated in a 96-well plate (1×10^4 cells/well) with 100 µL DMEM and incubated for 24 h at 37 °C. Res at different doses (50, 100, 200 µM) was added to each well and incubated for 30 min. The cells were then washed twice with CIB (calcium imaging buffer; 125 mM NaCl, 3 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 mM Fluo-3 AM, 0.1% (w/v) F-127, diluted with CIB) at 37 °C for 30 min. Cells were then washed twice with CIB and used immediately for imaging. We captured 120 graphs of the cells under blue light (1 s per picture) using an inverted fluorescent microscope (Nikon, Ti-U, Japan).

2.11. Body temperature detection

All the mice were housed in the procedure area the day before the injection. The mice were randomly divided into 3 groups. The mice were given Res (0, 10 mg/kg) by intragastric administration (i.g.) and then placed in a restrainer. After 30 mins, injected intravenously (i.v.) with C48/80 (2.5 mg/kg). The body temperature was recorded using a biological function experimental system, in which a probe was inserted into the anus of the mice for 30 min.

2.12. Hind paw swelling and extravasation

Thirty mins after given Res (5, 10, 20 mg/kg) by i.g., mice were anaesthetized and injected i.v. with 0.2 mL of 0.4% Evans blue in saline. A Vernier caliper was used to measure the thickness of the paw before any injection and as needed during the experiments. Five minutes later, 30 µg/mL C48/80 in saline was injected into the left paw and saline only into the right paw as a negative control. After 15 min, paw thickness was measured again and recorded. The mice were killed, the paws were separated and cut into small pieces and collected, dried at 50 °C, and weighed. Evans Blue was subsequently extracted by adding 500 µL acetone-saline (7:3) mixture 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.

2.13. Histological analysis

Mice were treated followed the Hind paw swelling method, 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. The sectional area of the blood vessels was measured by using the Image J software [27,28]. For avidin staining, the slides were dried at 37 °C for 30 min and pre-incubated in 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 3 times with PBS, and a drop of Fluoro-mount G (Southern Biotech, AL, U.S.A) was added. Degranulated MCs were identified by the appearance of green fluorescence granules outside of MCs with a lower content of condensed intracellular granules [29]. Images were taken immediately using a fluorescence microscope (Nikon, Tokyo, Japan).

2.14. Western blot

Protein was extracted from LAD2 cells treated with Res 24 h and C48/80 6 h, or SFN 24 h and C48/80 6 h. Proteins levels were detected with Western blot. Briefly, cells were prepared by extracting proteins with RIPA lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail on ice. The cell lysates were denatured by boiling in 5 × loading sample buffer for 5 min and separated by 10% SDS-PAGE. Following electrophoresis, the separated proteins were transferred to polyvinylidene fluoride membranes and blocked. The membranes were then incubated with specific primary antibodies at 4 °C for 8 h, including GAPDH (1:5000) and MRGPRX2 (1:1000), followed by incubation with secondary antibodies for 1 h 37 °C. The membranes were then developed using an enhanced chemiluminescence kit. A Lane 1DTM transilluminator was used to image the developed blots, and Image-Pro Plus 5.1 software was used to quantify the protein levels.

2.15. Quantitative real-time PCR

LAD2 cells (2×10^6 /well in a 6-well plate) were incubated with Res (200 µM) and SFN (5 µM) for 24 h and then stimulated with C48/80 (30 µg/mL) for 6 h at 37 °C [30]. Total RNA was isolated using Trizol extraction kit according to the manufacturer's instructions. Quantitative real-time PCR was performed on the CFX Connect™ Real-Time PCR

(BIO-RAD, USA). Relative quantities were estimated by the $\Delta\Delta\text{-Czt}$ method.

2.16. Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD) and were statistically analyzed using analysis of variance (ANOVA). Differences were considered significant at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The correlation test was undertaken with the person product-moment correlation.

3. Results

3.1. Res reduced C48/80-induced calcium flux in LAD2 cells and MPMC cells

The CCK8 assay was used to examine the cell toxicity of Res, there was no cytotoxicity in the Res concentration range from 6.25 to 400 μM (Fig. 1B). Intracellular Ca^{2+} level increase has been proposed as an essential trigger for MCs activation [31]. To determine the effect of Res on calcium flux of MCs, LAD2 cells or MPMC cells were pretreated with Res (50, 100, 200 μM) in Fluo-3 AM and analyzed by flow cytometry before and after C48/80 stimulation. Res induced a dose-dependent decrease in the C48/80-triggered calcium flux in LAD2 cells (Fig. 1C) and MPMCs (Fig. 1D).

3.2. Res decreased C48/80-induced degranulation in LAD2 cells

To evaluate the effect of Res on MCs degranulation and inflammation factor releasing, the secretion of β -hexosaminidase, histamine, TNF- α , MCP-1 and IL-8 in LAD2 cells were detected after Res and C48/80 treatment. Res inhibited the release of β -hexosaminidase in a dose-dependent manner (Fig. 2A), with IC_{50} value of $77.03 \pm 3.12 \mu\text{M}$ (Fig. 2B). This inhibition of degranulation by Res was confirmed based on the reduced secretion of histamines from Res-treated LAD2 cells (Fig. 2C). Mast cell-derived pro-inflammatory cytokines, especially TNF- α , MCP-1 and IL-8 have a critical biological role for inducing delayed type hypersensitive allergic and inflammatory responses [32]. These cytokines were all elevated by C48/80, but was suppressed by Res in a dose-dependent manner (Fig. 2D-F).

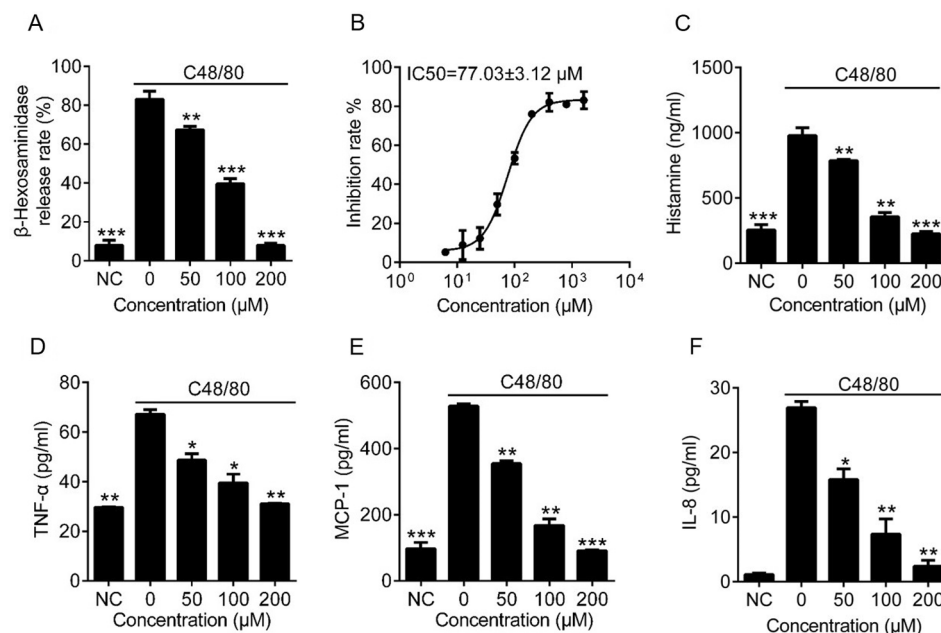


Fig. 2. Res inhibits C48/80-induced mast cell degranulation. A-B. C48/80 was used as an agonist for LAD2 cells. Tyrode's solution was used as a negative control. Release of β -hexosaminidase (A) and histamine (B) from LAD2 cells treated with Res (0, 50, 100 or 200 μM) and C48/80 (30 $\mu\text{g/mL}$) for 30 min. C. The IC_{50} value of Res for β -hexosaminidase secretion. D-F. Release of MCP-1 (D), IL-8 (E) and TNF- α (F) from LAD2 cells treated with Res (0, 50, 100 or 200 μM) and C48/80 (30 $\mu\text{g/mL}$) for 6 h. Data are presented as mean $\pm$ S. D. and are representative of three independent experiments with three samples per experiment. ANOVA was used to determine significance in statistical comparisons (* $P < .05$, ** $P < .01$ and *** $P < .001$ compared with 0 group).

We also measured the effect of Res on IgE-mediated MCs activation. Streptavidin induced degranulation in IgE-sensitized MCs, and pretreatment with Res (5, 10, and 20 μM) significantly inhibited the release of β -hexosaminidase (Supplementary figure 1A), with IC_{50} value of $10.38 \pm 0.98 \mu\text{M}$ (Supplementary figure 1B). Histamine release was also inhibited in a dose-dependent manner (Supplementary figure 1C). The production of TNF- α , LTB4, and MCP-1 were examined, and the release of these mediators was dose-dependently reduced by Res (Supplementary figure 1D-F).

3.3. Res suppressed C48/80-induced local inflammation

Next, we demonstrated the inhibition effects of Res on C48/80-evoked hand paw inflammation *in vivo*. Thirty mins before injection C48/80 into left paws, mice were intragastrically administrated with Res (5, 10, 20 mg/kg). The paw thickness and Evans blue stained extravasation leakage into the paw were investigated to characterize the pseudo-anaphylactic response. As showed in Fig. 3A, Res suppressed C48/80-induced extravasation and swelling in mice in a dose-dependent manner. We next detected skin vascular permeability of the related paw skin sections paw by H&E and determined the sectional area of blood vessel, the results showed that Res treatment reduced the hemangiectasis (Fig. 3B). Furthermore, we studied whether the inhibition effect of Res in hind paw inflammation and hemangiectasis was associated with MCs by avidin staining test. The results showed that Res also decreased the number of degranulated MCs (Fig. 3C). We next determined the sectional area of blood vessel in Fig. 3B and the degranulation rate of MCs in Fig. 3C. The correlation analysis showed that vascular permeability and MCs activation are strongly correlated (person $r = 0.9441545$, $P = 0.0157$, and 95% confidence interval 0.3705782, 0.9964130), which suggested that Res inhibits increased vascular permeability by suppressing MCs activation (Fig. 3D).

3.4. Res inhibited C48/80-induced systemic anaphylaxis

Having proved that Res can inhibit the MCs-activation *in vitro*, we next determined the effects of Res on anaphylaxis by measuring the C48/80-induced drop in body temperature in mice before and after i.g. administration of Res (10 mg/kg). The results showed that C48/80 induced a rapid drop in body temperature ($3.56 \pm 0.40 ^\circ\text{C}$) that was very

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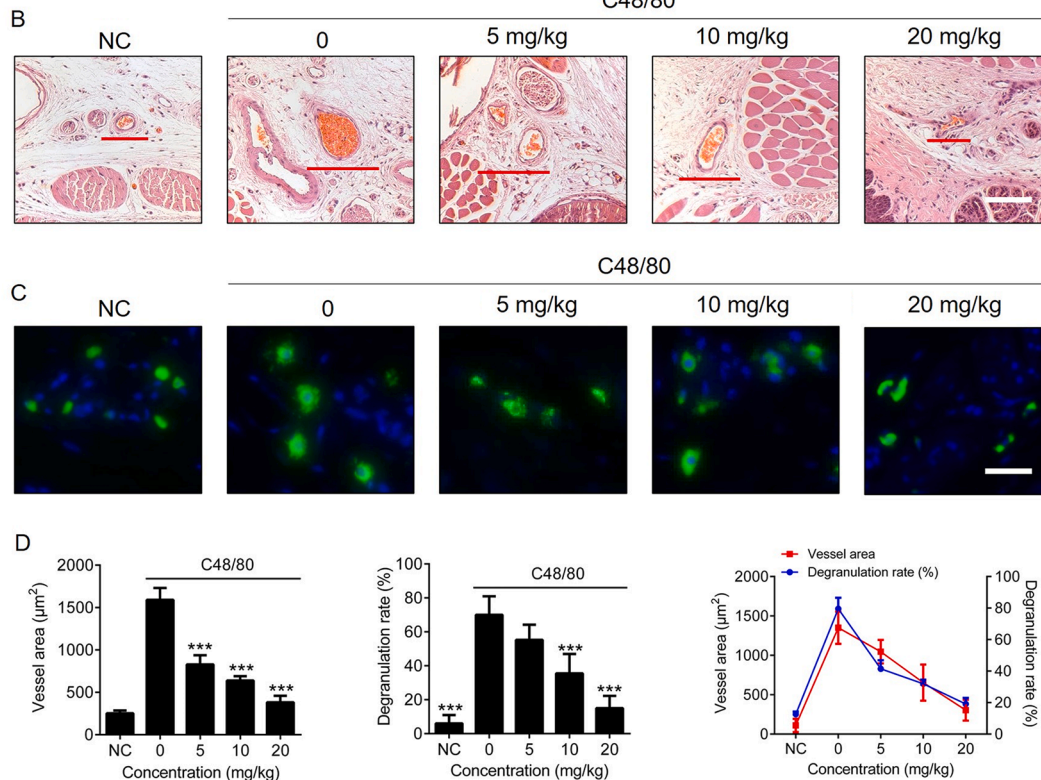
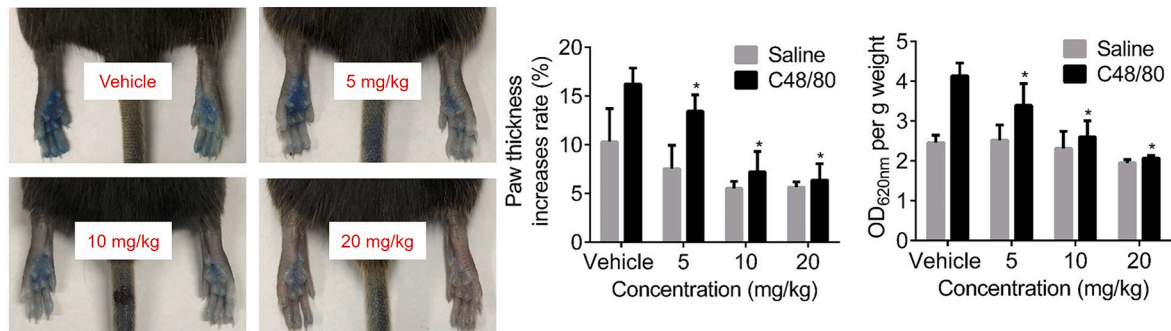


Fig. 3. Res suppresses C48/80-induced PCA and mast cell degranulation. A. Different Res concentrations (0, 5, 10, 20 mg/kg) or saline was orally administered to mice. At 30 min post-treatment, 30 $\mu\text{g}/\text{mL}$ C48/80 in saline was injected into the left paw, while saline was simultaneously injected into the right paw as a vehicle control. The degree of swelling and Evans blue exudation in mice hind paws were analyzed 15 min after injection. B. H&E staining of the paw skin (The red line indicated the blood vessel and red blood cells inside it) C. Avidin staining of the paw skin (scale bar = 50 μm). D. The quantification of the sectional area of the blood vessels, the quantification of degranulation rate of MCs in skin and the correlation analysis between vascular permeability (vessel area μm^2) and MCs activation (Degranulation rate %). Data are presented as mean $\pm$ S. D. and are representative of three independent experiments with seven mice per experiment. ANOVA was used to determine significance in statistical comparisons (* P < .05 compared with left paw, *** P < .001 compared with 0 group).

slow to recover (more than 30 mins after injection of C48/80), while Res-treated mice showed a much smaller drop (1.6 ± 0.32 °C) and recovered quickly (18mins after injection of C48/80) (Fig. 4A). This was probably because the inhibition of histamine release in serum levels (measured 30 mins after injection of C48/80) (Fig. 4B) [33]. Res also inhibited the serum levels of TNF- α , IL-8 and MCP-1 (measured 4 h after injection of C48/80) in a dose-dependent manner (Fig. 4C-E).

3.5. Res promoted the expression of Nrf2 and HO-1 in LAD2 cells

Nrf2 activation by Res was investigated in LAD2 cells by using western blot and PCR. Increased Nrf2 protein expression (Fig. 5A) and transcriptional activity (Fig. 5B) were observed following Res and SFN 24 h or C48/80 6 h in LAD2 cells. SFN, the well-established Nrf2 activator, served as positive control [34]. Furthermore, we asked whether

the expression of known Nrf2 target genes is modulated by Res treatment. The results showed that Res significantly increased transcription of the Nrf2 target genes HO-1 and NQO1 (Fig. 5C-D). Treatment with C48/80 significantly up-regulated mRNA expression of the pro-inflammatory cytokine PGD₂, MCP-1, TNF- α , and IL-1 β , while pre-treatment with Res prior C48/80 significantly reduced the levels of these cytokines (Fig. 5E-H). Since C48/80-induced MCs activation is mediated by MRGPRX2, we detected whether the expression of MRGPRX2 is modulated by Res treatment. The results showed that C48/80 up-regulated mRNA expression of MRGPRX2, while pre-treatment with Res and SFN prior C48/80 significantly reduced the level of MRGPRX2 (Fig. 5I).

We also measured whether Res modulated IgE-induced mRNA expression of pro-inflammatory cytokine TNF- α and MCP-1 in MCs. IgE/Streptavidin significantly up-regulated mRNA expression of TNF- α and

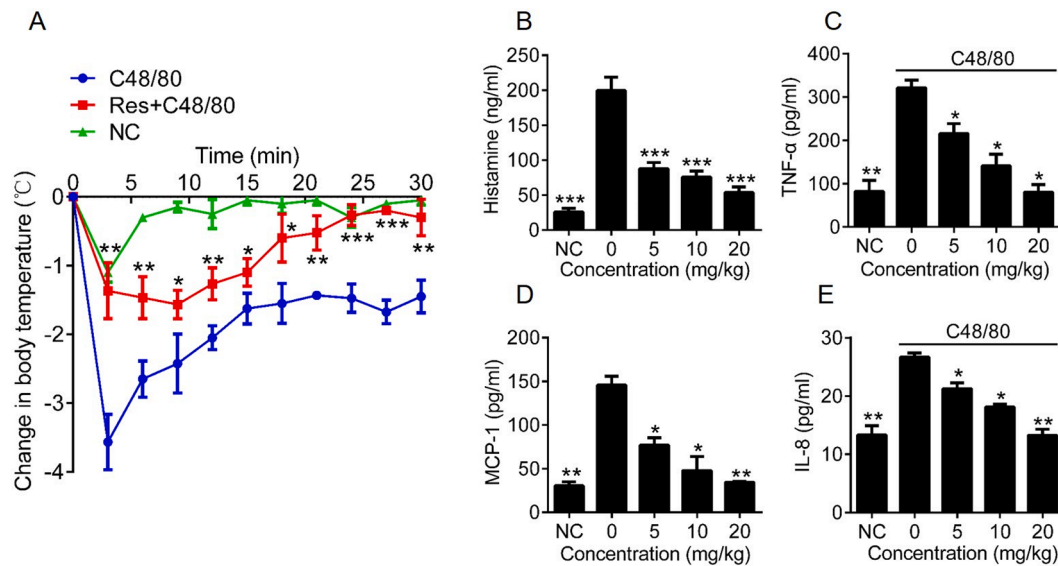


Fig. 4. Res suppresses C48/80-induced systemic anaphylactic reaction. A. Mice were pretreated with Res (10 mg/kg) or saline, change in body temperature was measured 30 min after injection with C48/80 into the tail vein. B-E. Mice were pretreated with different Res concentrations (0, 5, 10, 20 mg/kg) or saline. Serum levels of histamine (B), TNF-α (C), MCP-1 (D), and IL-8 (E) were measured after injection with C48/80 into the tail vein. Data are presented as mean ± S.D. and are representative of three independent experiments with seven mice per experiment. ANOVA was used to determine significance in statistical comparisons (* $P < .05$, ** $P < .01$ and *** $P < .001$ compared with injection of C48/80 and saline).

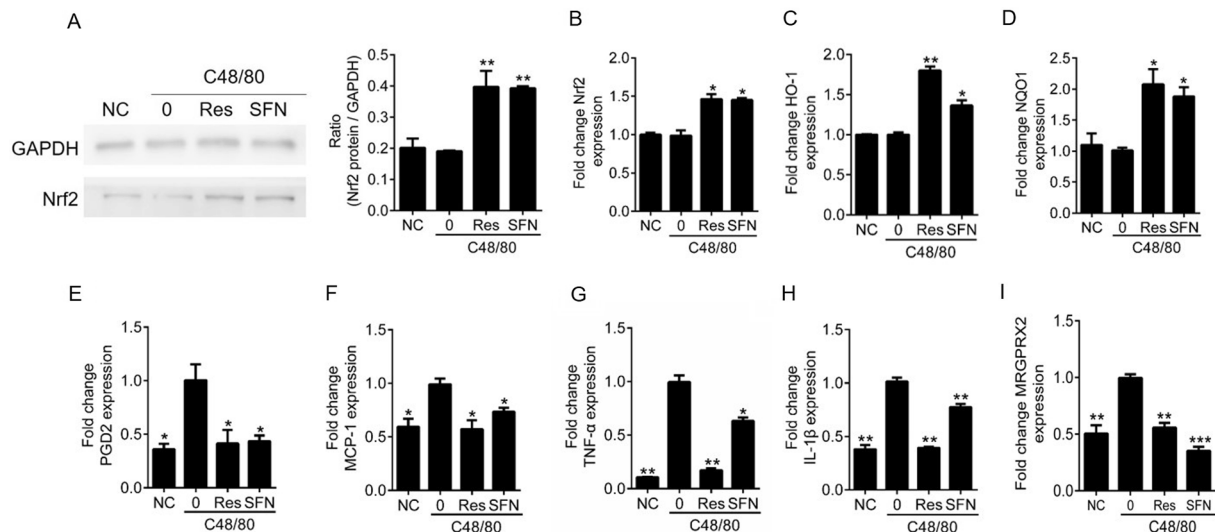


Fig. 5. Res affects the expression of Nrf2 and MCs target genes in human LAD2 cells. LAD2 cells treated with Res 24 h and C48/80 6 h, or SFN 24 h and C48/80 6 h. (A) The level of Nrf2 protein expression by western blot and quantification of Nrf2 protein expression by densitometric analysis. (B) Nrf2, (C) HO-1, (D) NQO1, (E) PGD2, (F) MCP-1, (G) TNF-α, (H) IL-1β, (I) MRGPRX2 in LAD2 cells were measured by q RT-PCR and data reported as fold changes in normalized expression. Data are presented as mean ± S.D. and are representative of three independent experiments with three samples per experiment. ANOVA was used to determine significance in statistical comparisons (* $P < .05$ and ** $P < .01$ compared with 0 group).

MCP-1, whilst pre-treatment with Res and SFN reduced levels of TNF-α and MCP-1 (Supplementary figure 2).

3.6. Nrf2 inhibitor abolished the protective effect of Res on MCs

To assess whether the Nrf2/HO-1 contributed to the MRGPRX2-mediated MCs activation, we inhibited Nrf2 activation by using a potent specific inhibitor ML385 in LAD2 cells [35]. It was showed that under the treatment of ML385, the inhibition effects of Res and SFN on C48/80-induced MCs activation were weakened, including β-hexosaminidase (Fig. 6A) and histamine release (Fig. 6B). ML385 also abolished the reduction of Res and SFN on C48/80-induced Ca^{2+} in flux in LAD2 cells (Fig. 6C). Furthermore, the results of RT-PCR analysis

showed that ML385 downregulated Nrf2 expression (Fig. 6D) and decreased the inhibitory effect of Res on HO-1 and NQO1 (Fig. 6E-F). In addition, Res inhibited the C48/80-induced mRNA expression of the pro-inflammatory cytokine, while these effects were completely abolished in the presence of ML385 (Fig. 6G-J).

4. Discussion

MCs, which are best known for causing a variety of symptoms in allergic reactions, generate and release the pro-inflammatory mediators in a process termed degranulation [36]. In addition to the FcεRI, it is apparent that MCs are also activated through non-IgE receptors, including MRGPRX2, the much more significance of which in asthma,

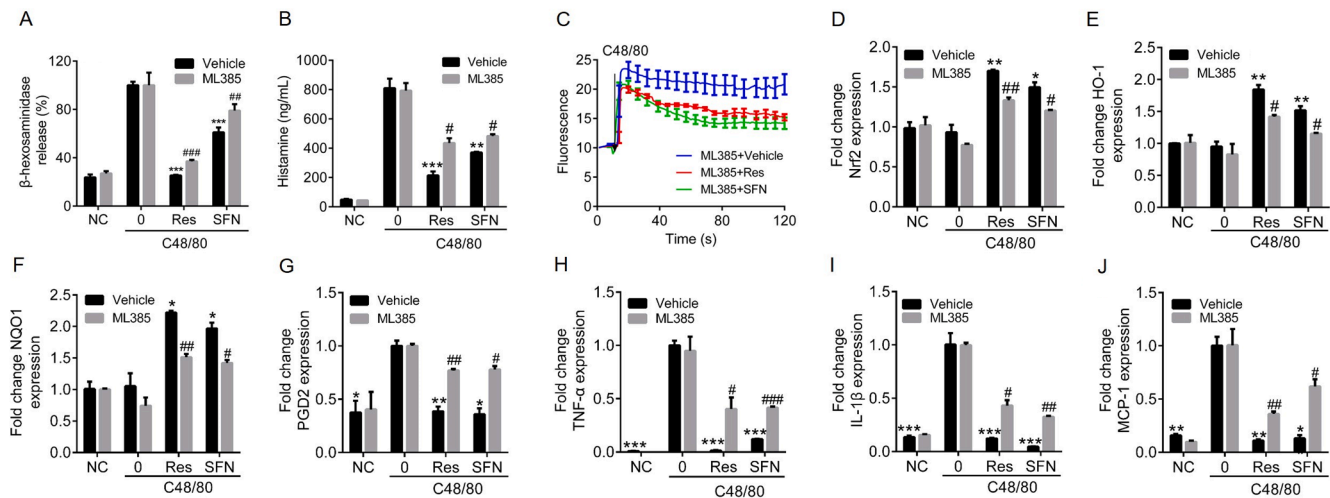


Fig. 6. Nrf2 inhibition attenuates the protective effects of Res on C48/80-induced secretion of mediators. LAD2 cells were pre-treated with the Nrf2 inhibitor ML385 (5 μ M) or vehicle for 72 h. Release of (A) β -hexosaminidase and (B) Histamine from LAD2 cells treated with Res (200 μ M) and C48/80 for 30 min. (C) Representative Ca^{2+} imaging traces in LAD2 cells pretreated with ML385 in response to 200 μ M Res and 30 μ g/mL C48/80. (D) Nrf2, (E) HO-1, (F) NQO1, (G) PGD2, (H) TNF- α , (I) IL-1 β , (J) MCP-1 in LAD2 cells were measured by q RT-PCR and data reported as fold changes in normalized expression. Data are presented as mean $\pm$ S.D. and are representative of three independent experiments with three samples per experiment. ANOVA was used to determine significance in statistical comparisons (* P < .05, ** P < .01 and *** P < .001 compared with 0 group, # P < .05, ## P < .01 and ### P < .001 compared with each vehicle group).

urticaria, and anaphylaxis is just beginning to be understood [8]. Therefore, there is an urgent need for investigating the inhibitory mechanisms in pseudo-allergy involving MRGPRX2-mediated MC activation.

Although allergy Fc ϵ RI- and pseudo-allergic MRGPRX2-triggered MCs activation routes are completely independent from each other [31], an inhibitory receptor CD300f is found to regulate both IgE-dependent and IgE-independent anaphylactic responses [32]. Furthermore, Res and Polydatin have been suggested to show the inhibitory effects in IgE-mediated MCs activation and Polydatin also has been reported to up-regulate Nrf2/HO-1 pathway in MCs [37]. Based on these previous studies, we speculated that Res up-regulates the signaling pathway of Nrf2/HO-1 to inhibit MRGPRX2-mediated pseudo-allergic reactions.

The rise in calcium levels is critical to MRGPRX2-mediated MC degranulation [18,19]. The results revealed that Res dose-dependently suppress calcium flux increase when added C48/80 into LAD2 cells, which was equally effective in MPMC. β -hexosaminidase and histamine release are the marks of MCs degranulation, so we use histamine and β -hexosaminidase assay in LAD2 cells to measure the protective effects of Res. The results indicated that Res inhibited the C48/80-induced MCs activation without causing cytotoxic effects. We also measured the effect of Res on IgE-dependent LAD2 cells activation, and Res showed rather higher inhibitory effect (IC_{50} value was 10.38 ± 0.95 μ M) than that on C48/80-induced MCs activation (IC_{50} value was 77.03 ± 3.12 μ M).

In human subjects, most MCs that are found in connective tissues, such as the skin [8]. Therefore, we used PCA model to measure the Res effects *in vivo*. The results showed that the content of Evans blue and the swelling of the left hind paw in mice were reduced by Res treatment. The hind paw sections were stained with H&E and avidin, which further proved that the diminished allergy symptoms are positively associated with the inhibition effects of Res on MCs activation, especially the release of histamine which is benefit for the vasodilation and increased vascular permeability [38].

The released histamine produced hypothermia via histamine H (1) receptors after i. v injection in mice with C48/80 [39]. In our study, Res helped the mice recover the C48/80-induced decrease in body temperature, which might due to the inhibition of the histamine releasing in plasma. The levels of some cytokines are elevated in serum during anaphylaxis in human beings and are associated with severe clinical symptoms [40]. The results showed that the levels of TNF- α , IL-8 and

MCP-1 were dramatically decreased following Res administration.

In our study, Res also promoted the expression of Nrf2 protein in MCs, and upregulated expression of transcription factor HO-1 and NQO1. Manipulation of the Nrf2/HO-1 pathways have been shown to protect cells under oxidative damage and inflammation conditions [41]. Targeting Nrf2 with pharmacologically-active compounds could modulate SP-induced primary human MCs activation [30]. In our study, Res up-regulated the protein expression and mRNA level of Nrf2. Activated Nrf2 translocated into the nucleus and binds to the antioxidant response element (ARE), which initiates transcription of multiple downstream anti-oxidant genes, such as HO-1. HO-1 activity has recently attracted significant attention due to its apparent involvement in modulation of the inflammatory response. Hemin, an inducer of HO-1, attenuated C48/80-elicited degranulation of MCs [42]. Bilirubin, one of the products of HO-1 might be involved in anti-allergic activity in MCs [42]. Moreover, HO-1-derived CO also reduced the production of proinflammatory cytokines such as TNF- α [43]. Our results showed that Res induced HO-1 expression in mRNA, which associated with its inhibitory effect on MCs activation. Furthermore, the activation of HO-1 has a significant protective effect against airway inflammation and hypersensitivity, which may demonstrate the anti-asthmatic effect of Res [44]. Importantly, the Nrf-2 inhibitor, ML385, attenuated the inhibitory effect of Res and SFN on inflammatory mediator gene expression. Consistently, its downstream targets HO-1 and NQO1 were also decreased by ML385. These data suggest that the anti-allergic effects of Res depend on the presence of Nrf2. We further detected whether the expression of MRGPRX2 is modulated by Res treatment. The results showed that pre-treatment with Res prior C48/80 significantly reduced the mRNA level of MRGPRX2. Therefore, Nrf2 seems to be a downstream effect of Res, Res may alter the expression of MRGPRX2.

In conclusion, the present study showed that Res exerts an inhibitory effect on MRGPRX2-mediated MCs activation by targeting Nrf2 pathway. Res may present a promising new therapeutic agent for the treatment of drug-induced anaphylactoid reactions and MC-dependent allergic disorders.

CRediT authorship contribution statement

Jue Wang: Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Yongjing Zhang:** Investigation, Data

curation. **Shiling Hu:** Investigation. **Shuai Ge:** Investigation. **Min Jia:** Supervision, Software. **Nan Wang:** Conceptualization, Methodology, Supervision, Project administration, Validation, Funding acquisition.

Declaration of Competing Interest

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.

Acknowledgment

This work was cofounded by National Natural Science Foundation of China (Grant number: 81930096), China Postdoctoral Science Foundation (Grant number: 2019M653672), Fundamental Research Funds for the Central Universities, China (Grant number: xjj2018168) and the Science and Technology Innovation Base-Open and Sharing Platform of Science and Technology Resources Project of Shaanxi Province, China (Grant number: 2019PT-26).

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2021.107426>.

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SUPPLEMENTARY MATERIALS

Figure S1. Res suppresses streptavidin-induced LAD2 cell degranulation.

Figure S2. Res affects the streptavidin-induced expression of target genes in human LAD2 cells.

Figure S1. Res suppresses streptavidin-induced LAD2 cell degranulation. Tyrode's solution was used as a negative control (NC). LAD2 cells were pre-sensitized with IgE (100 ng/mL) for 24 h. A. and B. Release of β -hexosaminidase (A) and histamine (B) from LAD2 cells treated with Res (0, 5, 10, and 20 μ M) and streptavidin (100 ng/mL) for 30 min. C. The IC₅₀ value of Res for β -hexosaminidase secretion. D–F. Release of TNF- α (D), IL-6 (E), and LTB₄ (F) from LAD2 cells treated with ISO (0, 50, 100, and 200 μ M) and streptavidin (100 ng/mL) for 6 h. * P < 0.05, ** P < 0.01, and *** P < 0.001 compared with the 0 μ M group (n = 3).

Figure S2. Res affects the streptavidin-induced expression of target genes in human LAD2 cells. LAD2 cells were pre-sensitized with IgE (100 ng/mL) for 24 h. (D)TNF- α , (E) MCP-1 in LAD2 cells treated with Res (20 μ M) for 24 h and streptavidin for 6 h, or SFN for 24 h and streptavidin for 6 h were measured by q RT-PCR and data reported as fold changes in normalized expression. * P < 0.05, and ** P < 0.01, compared with the 0 μ M group (n = 3).