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Inhibitory effect of daphnetin on the C48/80-induced pseudo-allergic reaction

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ABSTRACT

Pseudo-allergic reaction is an allergic reaction mediated by nonimmunoglobulin E (IgE), which does not require prior contact with antigen sensitization and directly leads to mast cell degranulation. Daphnetin (DAP) is known for its anti-inflammatory effects, but there are few studies on the effect of DAP on pseudo-allergy and its mechanism. To investigate the effect of DAP on pseudo-allergy and its mechanism, we inflicted pseudo-allergy on RBL-2H3 cells using C48/80 in vitro. Moreover, to assess the antipseudo-allergy effect of C48/80 in vivo, mouse models of local anaphylaxis, systemic anaphylaxis, and itch were used. The in vitro results show that DAP inhibits degranulation and chemokine release; furthermore, DAP reduced the activation of the PLC-IP3R and MAPK signaling pathways induced by C48/80. Additionally, our in vivo results showed that DAP inhibited C48/80-induced local anaphylaxis and inhibited eosinophil aggregation, vasodilation and mast cell degranulation. In systemic anaphylaxis, DAP inhibits the decrease in body temperature and reduces the release of His, TNF-a and IL-8. In C48/80-induced itch, the number of scratches in mice was reduced. Our results demonstrate that DAP can play a suppressive role in the pseudo-allergy induced by C48/80, providing information for the cure of disorders linked to pseudo-allergic reactions.

1. Introduction

With the development of industrialization, changes in environmental factors, the adjustment of people's diet structure and the increase in exposure to allergens, the incidence of allergic diseases continues to rise [1]. While immunoglobulin E (IgE)-type hypersensitivity is typically associated with allergic disorders, pseudo-allergies have a significant role in allergic illnesses as well [2–4]. Pseudo-allergic reactions can occur at the first time of administration [5,6], showing similar symptoms to IgE-type hypersensitivity in clinical practice, which can cause symptoms such as nausea, dermatitis, hypotension, and allergic shock.

Mast cells (MCs) play a central role in inflammation and allergies [7]. They may release inflammatory mediators, such as histamine (His), leading to allergic reactions [8,9]. Some stimulators, such as C48/80 and hymenopteran venoms, directly stimulate mast cells through nonimmune stimulation [10]. Compound 48/80 is a crosslinked condensation product of phenylethylamine and formaldehyde. This molecule is considered one of the most effective mast cell secretagogues in biochemical studies and is the most commonly used stimulator in pseudo-allergic studies. Compound 48/80 can strongly activate cell exocytosis and lead to rapid release of allergic mediators such as histamine in cells, thereby inducing pseudo-allergic reactions in the body. According to research, C48/80 triggers MRGPRX2/MrgprB2 to cause degranulation that is not dependent on IgE [11]. Once mast cells are activated, intracellular signaling pathways are affected, which can directly activate the trimer G protein and then send signals downstream to activate phospholipase C (PLC), phosphorylation of phospholipase Cγ, and intracellular calcium mobilization [12]. This affects the activation of more downstream molecules, such as IP3R, PKC, and MAPK, leading to the production of mast cell granules and inflammatory factors [13].

DAP, a 7,8-dihydroxycoumarin, is a natural substance with the safe and nontoxic properties of coumarin that has neuroprotective, anti-inflammatory, anti-malarial, and anti-allergic effects. [14–18]. However, few studies have revealed the effect of DAP on pseudo-allergy. Therefore, this study investigated the effect and mechanism of DAP on pseudo-allergic reactions.

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2. Materials and methods

2.1. Reagents

Daphnetin (DAP, purity ≥ 98%) was purchased from Chengdu Must Technology Co., Ltd. (Chengdu, Sichuan, China). Evans blue, MTT and Compound48/80 were supplied by Sigma-Aldrich (St. Louis, MO, USA). ELISA kits were from Shanghai FANKEL Industrial Co., Ltd. (Shanghai, China). Primary antibodies anti-PLCγ, anti-p-PLCγ, anti-p38, and anti-p-p38 were from Zen-Bioscience Co., Ltd. (Chengdu, Sichuan, China), anti-IP3R, anti-p-IP3R, anti-PKC, and anti-p-PKC were purchased from Cell Signaling Technology (Boston, MA, USA), and GAPDH, anti-ERK1/2, and anti-p-ERK1/2 were purchased from ABclonal Technology Co., Ltd. (Wuhan, Hubei, China). Secondary antibodies were from Beyotime Institute of Biotechnology (Shanghai, China).

2.2. Mice

Adult male ICR mice (20–22 g) were obtained from Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, Liaoning, China). The animals were kept in a pathogen-free environment with free access to food and water in an air-conditioned room. All animal studies were also approved by the Animal Ethics Committee of Jilin University (NO 20220062).

2.3. Cell lines

RBL-2H3 cells were obtained from the National Infrastructure of Cell Line Resource (Shanghai, China) and were kept in a 37 °C incubator with 5% CO_2 using MEM from HyClone (Logan, UT, USA), which contained 15% FBS and 1:100 penicillin–streptomycin.

2.4. Cytotoxicity assays

RBL-2H3 cells were grown in a 37 °C CO_2 incubator (2×10^5 cells/well in 96-well plates) for 24 h to measure cell cytotoxicity using MTT. The cells were then exposed to DAP for 24 h at various concentrations (0, 7.5, 15, 30, 60, and 120 μM). The cells were then exposed to 10% MTT for 4 h and dissolved in DMSO. Using a microplate reader, the vitality of the cells was assessed using the absorbance at 490 nm. (Biotek, Winusky, VT, USA).

Cell survival rate (%) = $[(OD_{Treated} - OD_{Blank})/(OD_{Control} - OD_{Blank})] \times 100\%$.

2.5. β-hexosaminidase assay

Forty-eight-well plates containing 2×10^5 RBL-2H3 cells were used for culture, which lasted 24 h. During a 30-minute incubation period with various doses of DAP (30, 60, and 120 μM), C48/80 (50 μg/mL) was used to activate the cells, and the supernatant was transferred to a 96-well plate. p-Nitrophenyl N-acetyl-D-glucosamide was also added in equal amounts. After incubation at 37 °C for 1.5 h, the termination solution was added to terminate the reaction, and the OD value on the microplate reader was used to quantitatively release the β-hexosaminidase.

2.6. Histamine and chemokine release assay

Forty-eight-well plates containing 2×10^5 RBL-2H3 cells were used for culture, which lasted 24 h. During a 30-minute incubation period with various doses of DAP (30, 60, and 120 μM), C48/80 (50 μg/mL) was used to activate the cells, and the supernatant was collected. His, IL-8 and TNF-α were detected by ELISA kits.

2.7. Toluidine blue staining

Forty-eight-well plates containing 2×10^5 RBL-2H3 cells were used for culture, which lasted 24 h. During a 30-minute incubation period with various doses of DAP (30, 60, and 120 μM), C48/80 (50 μg/mL) was used to activate the cells. After 30 min, the supernatant was discarded, and the cells were washed once with Tyrode's solution. Then, 4% paraformaldehyde was used to fix the cells. The surplus paraformaldehyde was rinsed with Tyrode's solution after the paraformaldehyde was discarded after 30 min. After staining with 1% toluidine blue for 35 min, the toluidine blue was discarded, and the cells were rinsed with distilled water for 1 min and observed under an inverted microscope.

2.8. F-actin structure analysis

six-well plates containing 2×10^5 RBL-2H3 cells were used for culture, which lasted 24 h. During a 30-minute incubation period with various doses of DAP (30, 60, and 120 μM), C48/80 (50 μg/mL) was used to activate the cells. After 30 min, the supernatant was discarded, and the cells were fixed with 4% paraformaldehyde for 30 min. The surplus paraformaldehyde was rinsed with Tyrode's solution after the paraformaldehyde was discarded. To improve permeability, 0.5% Triton X-100 solution was applied to the cells for 5 min. After washing the cells twice with Tyrode's solution, 1 × Fluor 488 phalloidin working solution was added and incubated for 60 min in the dark. The residue was washed with Tyrode's solution, and the coverslip was removed, dried, added to a sealant, sealed with a slide and observed under a fluorescence microscope.

2.9. Local anaphylaxis

Adult mice were randomly divided into five groups: control, model, DAP50 (50 mg/kg), DAP100 (100 mg/kg), and DAP200 (200 mg/kg). The DAP-treated groups were given DAP by intragastric administration, and the control and model groups were given the corresponding dose of 0.5% CMC-Na. One hour after administration on the 7th day, the mice were anesthetized, and C48/80 (30 μg/mL) was intradermally injected into the back skin of mice and injected subcutaneously into the left paw. The control group was given the corresponding volume of Tyrode's solution. Then, Evans blue solution was injected into the tail vein of mice. The thickness of paws was measured after 30 min. Mice were sacrificed, and paws and back skin tissues were collected. The thickness of back skin tissue was measured. Evans blue extraction was performed at 37 °C for 24 h in acetone-saline (7:3), and tissue homogenates were centrifuged (1000 g, 20 min). Two hundred microliters of the supernatant was evenly divided across 96-well cell culture plates. The absorbance at 620 nm was measured using a spectrophotometer.

2.10. Histological analysis

The back skin and paw tissues were removed, rinsed in PBS, fixed for 48 h, and stained with HE or toluidine blue. Images were captured using an optical microscope (Leica, Wetzlar, Germany).

2.11. Systemic anaphylaxis

Adult mice were randomly divided into five groups: control, model, DAP50 (50 mg/kg), DAP100 (100 mg/kg), and DAP200 (200 mg/kg). The DAP-treated groups were given DAP by intragastric administration for 7 days, and the control and model groups were given the corresponding dose of 0.5% CMC-Na. After 1 h of administration on the 7th day, the control group was injected with saline through the tail vein, and the mice were injected with C48/80 (0.3 mg/kg) through the tail vein, except for the control group. Body temperature was measured using an animal thermometer every 5 min for 30 min. The blood was centrifuged

for 15 min at 4 °C and 1000 g. The levels of His, TNF-α and IL-8 were detected by ELISA kits.

2.12. Scratching behavior test

Adult mice were divided into four groups at random: model, DAP-treated groups including DAP50 (50 mg/kg), DAP100 (100 mg/kg), and DAP200 (200 mg/kg), model group and DAP-treated groups were given DAP by intragastric administration, and C48/80 (1 mg/mL) was intradermally injected into the neck of mice 1 h after continuous administration for 7 days. The number and time of scratching were recorded immediately.

2.13. Western blot

Protein from RBL-2H3 cells was extracted under cold conditions using RIPA lysis buffer (Genstar, Beijing, China). This buffer contains protease inhibitors and phosphatase inhibitors. The protein concentration was measured with a kit (Beyotime, Shanghai, China). The protein was denatured by adding loading buffer to boil for 5 min, and the same amount of protein was separated by SDS-PAGE electrophoresis on a 10% gel. The isolated protein was transferred to a PVDF membrane and blocked for 2 h. The primary antibody was then incubated at 4 °C overnight, followed by three TBST washes every 10 min, the secondary antibody was incubated at room temperature for 1 h and then washed with TBST three times every 10 min at 25 °C. Exposure was performed in a darkroom. Data processing with ImageJ.

3. Results

3.1. Cytotoxicity assays

Cytotoxicity assays showed that DAP concentrations of 0, 7.5, 15, 30, 60, and 120 μM had no impact on the viability of RBL-2H3 cells (Fig. 1).

3.2. DAP inhibited C48/80-induced degranulation in RBL-2H3 cells

According to the DAP solubility and MTT assay results, we selected concentrations of 30, 60 and 120 μM to study the effect of DAP on RBL-2H3 cell degranulation. The release of β-Hex, His, IL-8 and TNF-α was chosen to confirm the effectiveness of DAP on C48/80-induced RBL-2H3 cell degranulation. The results showed that DAP could inhibit the release of β-Hex (Fig. 2A) and His (Fig. 2B). Likewise, IL-8 and TNF-α were suppressed by DAP (Fig. 2C and Fig. 2D). We also observed morphological changes in RBL-2H3 cells by toluidine blue staining. The results showed that with the increase of the dose of DAP , the cells were increasingly spindle-shaped or irregularly shaped, and the number of vacuole-shaped cells was reduced. As the dosage of DAP increased, the color of the outside of the cells progressively became shallow, the discharge of intracellular particles diminished, and the morphology gradually approached that of the control group (Fig. 3).

3.3. DAP inhibits C48/80-induced depolymerization of F-actin in RBL-2H3 cells

The cytoskeleton, a complex polymer network beneath the cell membrane, is essential for preserving the shape and functionality of cells in their native state. As a result, fundamental physiological changes in cells, such as inflammation, are constantly reflected in the cytoskeleton. It has been shown that the potential F-actin structure is crucial for mast cell activation. The changes in F-actin structure were observed by fluorescence microscopy. As shown in Fig. 4, RBL-2H3 cells in the control group showed a typical spindle shape, and F-actin was evenly distributed in the cells. After C48/80 stimulation, the cells contracted, F-actin depolymerized, and the cytoskeleton was destroyed. The DAP-treated groups inhibited the depolymerization of F-actin induced by C48/80 and gradually approached the control group with increasing administration dose.

3.4. DAP alleviated C48/80-induced local anaphylaxis

To determine the ability of DAP to reduce pseudo-allergy in vivo, C48/80 was injected into the back skin and paws of mice by intradermal injection and subcutaneous injection, respectively, and then intravenously injected with Evans blue. Representative images of the back skin and hind paws show a diminished inflammatory response of DAP to the back skin (Fig. 5A, Fig. 7A). DAP reduced the diameter of the blue spots on the back skin (Fig. 5B) and the paws swelling rate (Fig. 7B). Evans blue exudation was also inhibited (Fig. 5C, Fig. 7C). H&E staining of tissue sections showed that C48/80-triggered vasodilatation and eosinophil release were inhibited after DAP pretreatment (Fig. 6A-B, Fig. 8A-B). Toluidine blue staining showed that DAP significantly reduced mast cell degranulation in the back skins and paws (Fig. 6C, Fig. 8C).

3.5. DAP suppressed C48/80-triggered systemic anaphylaxis

To further evaluate the effect of DAP, we established a mouse model of systemic allergic reaction induced by C48/80. No mice died during the experiment. As shown in Fig. 9A, the temperature of mice decreased after injection of C48/80 and reached the lowest value at 15 min. DAP inhibited the decrease in body temperature and made the body temperature gradually reach that of the control group at 30 min. The concentrations of serum His (Fig. 9B), IL-8 (Fig. 9C) and TNF-a (Fig. 9D) triggered by C48/80 were decreased to some extent after DAP pretreatment. The above results further indicated that DAP alleviated the systemic anaphylaxis induced by C48/80 by inhibiting MC degranulation.

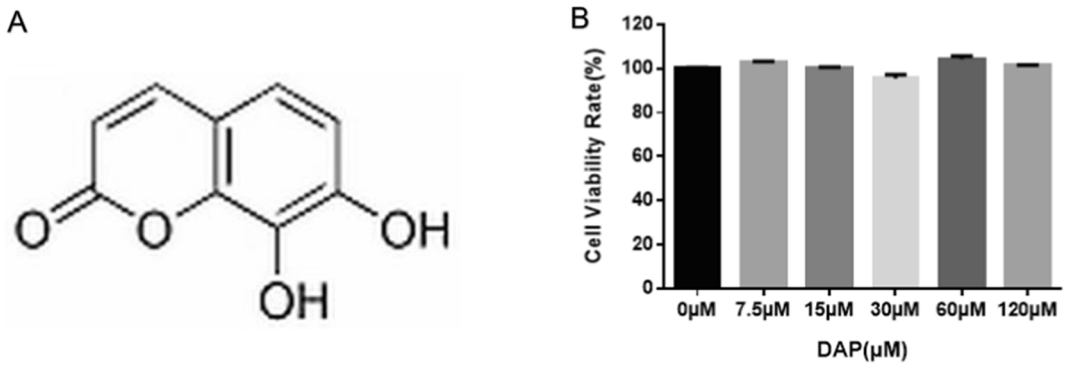

Fig. 1. Chemical structure of daphnetin and viability of DAP-treated on RBL-2H3 cells. A: Chemical structure of daphnetin (DAP) B: The cell viability in RBL-2H3 cells treated with 0, 7.5, 15, 30, 60, and 120 μM DAP. Data are presented as the mean ± SD, n = 3.

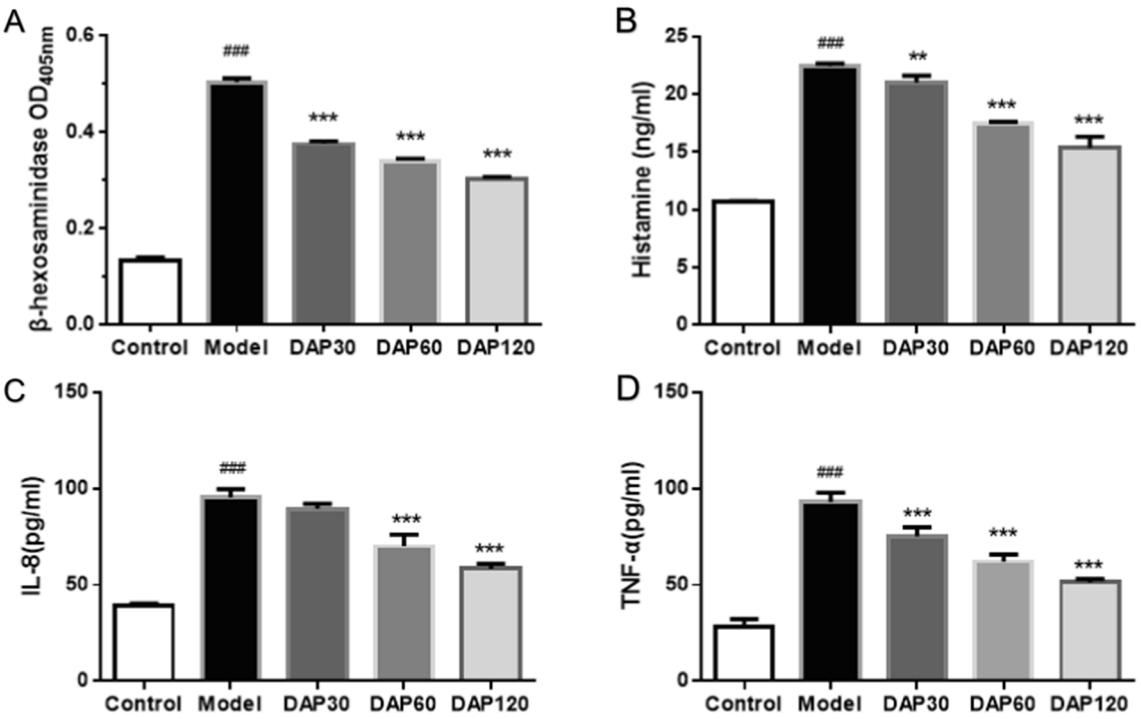

Fig. 2. DAP inhibited the release of β-Hex, His and inflammatory factors in RBL-2H3 cells induced by C48/80. A-B: DAP inhibits degranulation of RBL-2H3 cells to release β-Hex and His. C-D: DAP inhibits degranulation of RBL-2H3 cells to release IL-8(C) and TNF-α(D). Data are presented as the mean ± SD, n = 3. ANOVA was used to assess the significance of statistical comparisons. (###p < 0.001vs control group ; *P < 0.05, **P < 0.01, ***P < 0.001 vs model group.).

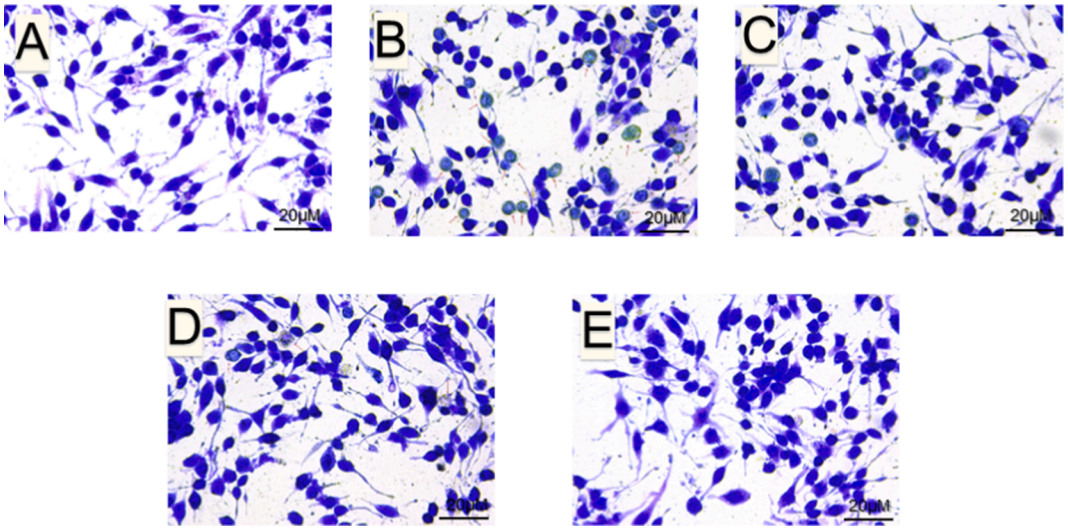

Fig. 3. DAP inhibited C48/80-induced morphological changes in RBL-2H3 cells. A: Control group: RBL-2H3 cells; B: Model group: C48/80-sensitized RBL-2H3 cells; C-E: DAP-treated group: Effect of treatment with DAP (30, 60, 120 μM) induced by C48/80 on RBL-2H3 cells, n = 3.

3.6. DAP suppressed scratching bouts induced by C48/80 in mice

Itch is a defining feature of allergic responses. We tested the ability of DAP to prevent mice from scratching when C48/80 was present. As shown in Fig. 10, when C48/80 was subcutaneously injected into the neck, the number of scratches in mice increased significantly in 30 min. In contrast, the number of scratches in mice decreased significantly within 30 min after DAP pretreatment (Fig. 10A). The scratching behavior was analyzed with an interval of 10 min, and it was found that the antipruritic effect of DAP was obvious within 20 min (Fig. 10B).

3.7. DAP inhibited the phosphorylation of PLCγ, IP3R, PKC, ERK1/2 and P38

After clarifying the inhibitory effect of DAP on pseudo-allergy, we studied its possible mechanism. Western blot analysis showed that C48/80 significantly increased the phosphorylation of PLCγ, IP3R, PKC, ERK1/2 and P38 in RBL-2H3 cells (Fig. 11, Fig. 12). After treatment with DAP, the phosphorylation of PLCγ, IP3R, PKC, ERK1/2 and P38 was significantly reduced.

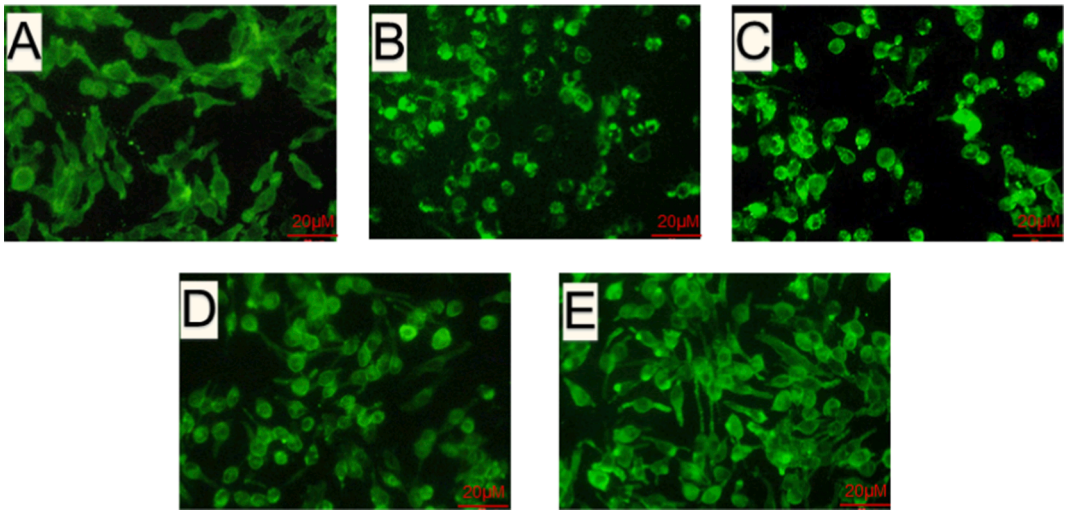

Fig. 4. DAP inhibits C48/80-induced depolymerization of F-actin in RBL-2H3 cells. A: Control group: RBL-2H3 cells; B: Model group: C48/80-sensitized RBL-2H3 cells; C-E: DAP-treated group: Effect of treatment with DAP (30, 60, 120 μM) induced by C48/80 on RBL-2H3 cells, n = 3.

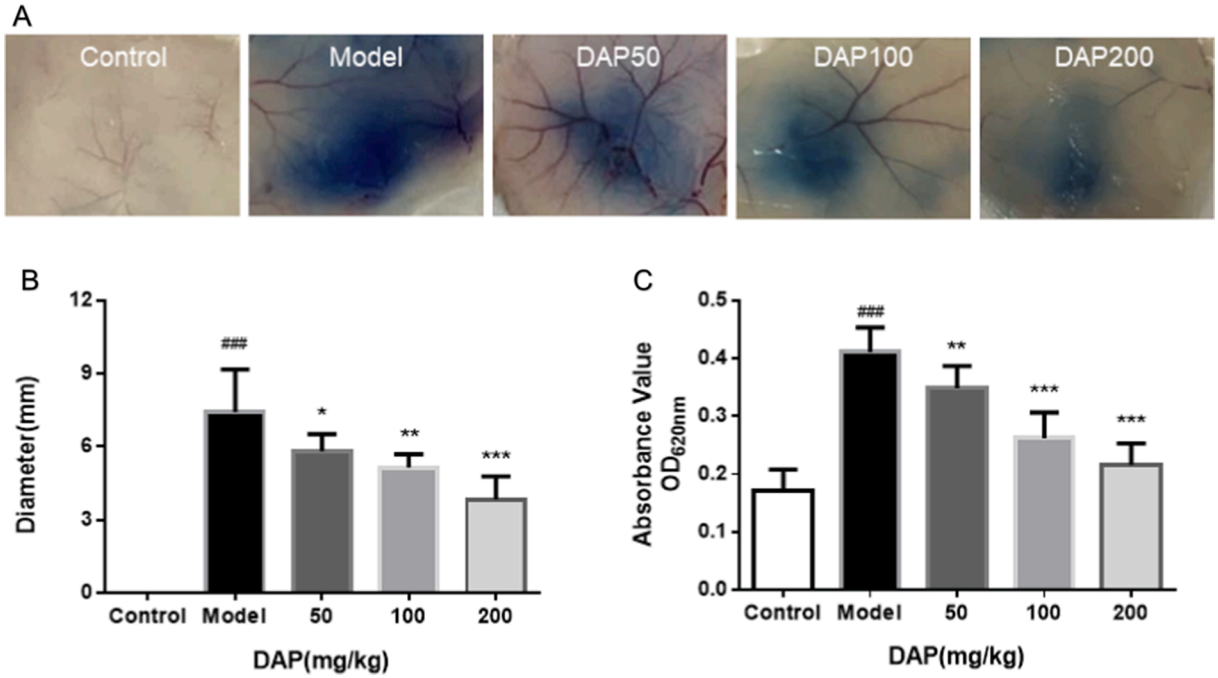

Fig. 5. DAP decreased C48/80-induced local anaphylaxis in mouse back skin. The treatment group was given DAP (0, 50, 100, and 200 mg/kg) and intradermal injection of 50 μg/ml C48/80 into the back skin. CMC-Na and intradermal injection of Tyrode's solution were given as controls. A: Representative images of Evans blue staining extravasation. B: Skin blue spot diameter. C: Quantification of Evans blue leaked into back skin. Data are presented as the mean ± SD, n = 6. ANOVA was used to assess the significance of statistical comparisons. (###p < 0.001vs control group ; *P < 0.05, **P < 0.01, ***P < 0.001 vs model group.).

4. Discussion

At present, most allergic reactions are based on IgE-mediated allergic reactions. In recent years, a new type of hypersensitivity has been observed. A non-IgE-mediated allergic reaction, that is, a pseudo-allergic reaction, is different from Type I allergy. This reaction does not require antigen mediation and directly causes mast cell activation, resulting in an allergic reaction in the body. Patients do not have high IgE levels. For this reaction, the first contact with allergic substances directly leads to the occurrence of allergic reactions. Common allergens in pseudo-allergy include some traditional Chinese medicine injections and anesthetics. The clinical symptoms are usually similar to IgE-mediated allergic reactions, including itching, dermatitis and other symptoms, and serious reactions can lead to allergic shock. Therefore, it is necessary to select effective candidate medicines for pseudo-allergy research.

Daphnetin, a common natural product, has been studied in anti-inflammatory, antioxidation, and antitumor studies, but research on its effect in pseudo-allergy has not been reported. Therefore, this study investigated the effect of daphnetin on C48/80-induced pseudo-allergy.

The mechanism of the pseudo-allergic reaction is complex, and some reports have proven the possible mechanism of C48/80-MRGPRX2-mediated MC activation. MCs have a significant impact on allergic responses in people with allergic disorders, according to previous studies [19]. RBL-2H3 cells have various biological characteristics of mast cells, which can serve as a standard model for researching mast cell

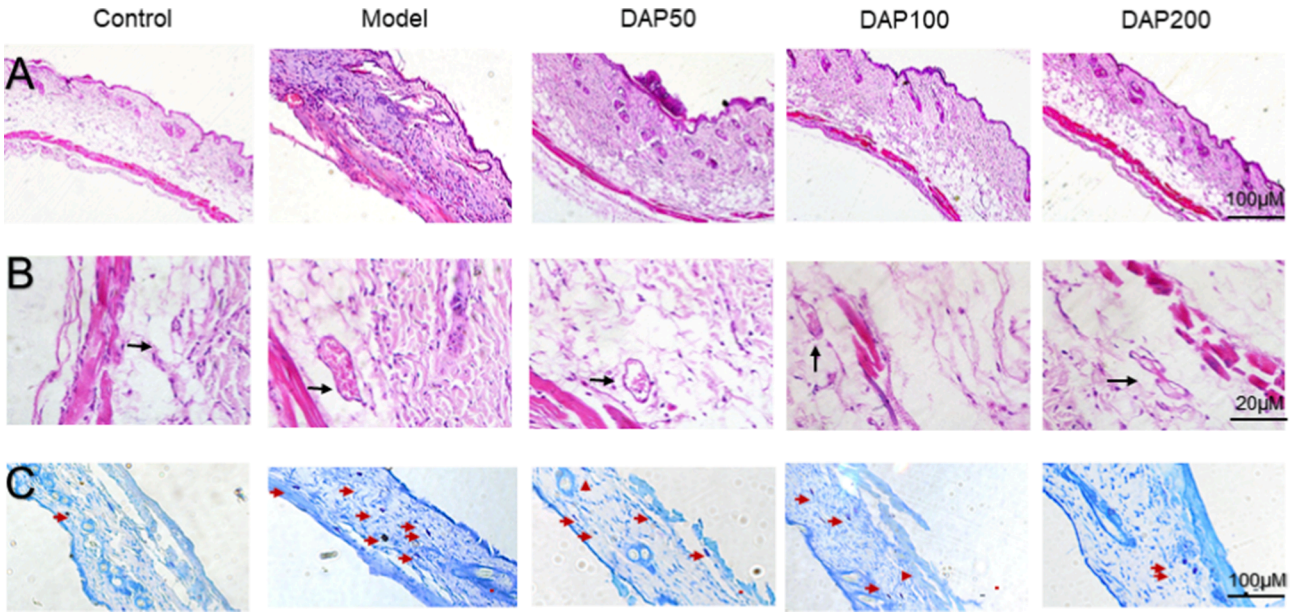

Fig. 6. DAP decreased C48/80-induced local anaphylaxis in mouse back skin by inhibiting MC degranulation. The treatment group was given DAP (0, 50, 100, and 200 mg/kg) and intradermal injection of 50 μg/ml C48/80 into the back skin. CMC-Na and intradermal injection of Tyrode's solution were given as controls, n = 4. A: DAP inhibits eosinophil release (H&E staining). B: DAP inhibited vasodilation (H&E staining). C: DAP inhibited mast cell degranulation (toluidine blue staining).

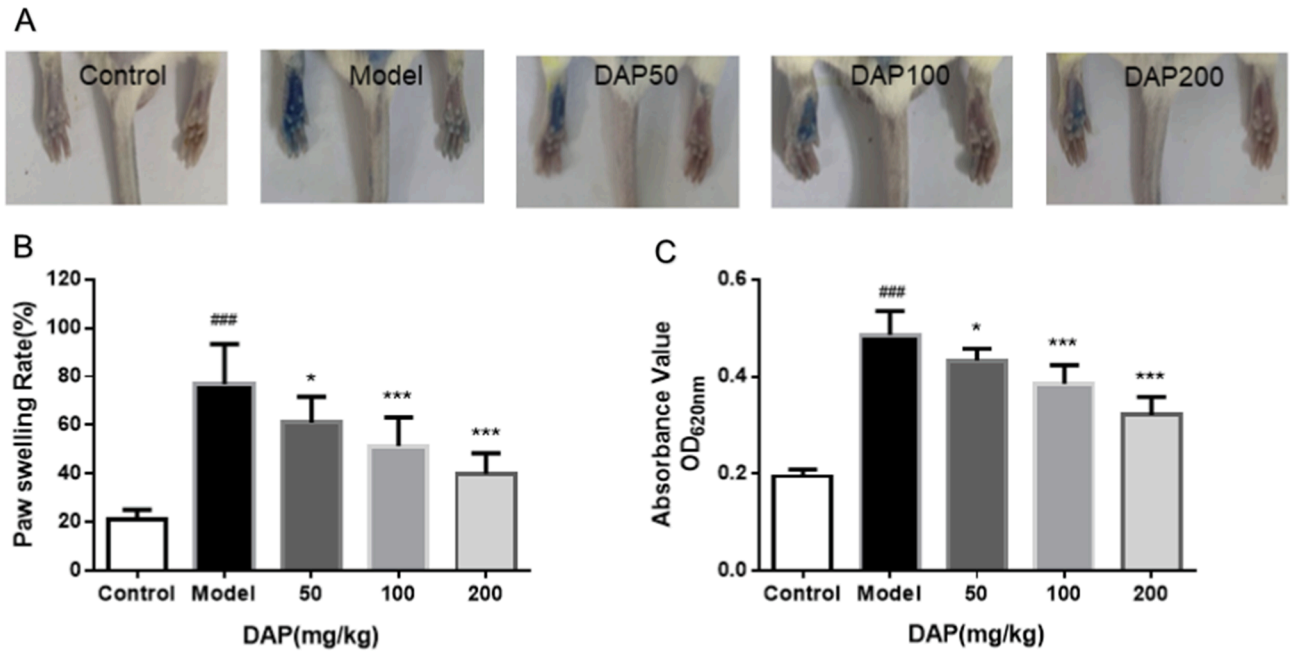

Fig. 7. DAP decreased C48/80-induced local anaphylaxis in mouse paw. The treatment group was given DAP (0, 50, 100, and 200 mg/kg) and subcutaneous injection of 50 μg/ml C48/80 into the paws. CMC-Na and subcutaneous injection of Tyrode's solution were given as controls. A:The representative images of Evans blue staining extravasation. B: Quantification of increased paw thickness (paw swelling rate) C: Quantification of Evans Blue Leaked into paws. Data are presented as the mean ± SD, n = 6. ANOVA was used to assess the significance of statistical comparisons. (###p < 0.001vs control group ; *P < 0.05, **P < 0.01, ***P < 0.001 vs model group.).

degranulation and are widely used in in vitro models of allergic diseases. Mast cell activators are frequently used to examine pseudo-allergic reactions, and the most popular activator is C48/80[20]. Therefore, we used a C48/80-induced RBL-2H3 cell degranulation in vitro model to verify the effect of DAP on C48/80-induced pseudo-allergy. When RBL-2H3 is stimulated to degranulate, some mediators, such as β-Hex and His, are released. β-Hex is a marker of mast cell degranulation. His release will cause vasodilation [21]. IL-8 is a chemical attractant for eosinophil and neutrophil recruitment. Likewise, the proinflammatory cytokine cascade is headed by the cytokine TNF-α [22,23]. In the present study, we found that DAP could inhibit the release of β-Hex, His, IL-8 and TNF-α. Toluidine blue dye can bind to substances in mast cells and can also bind to particles in mast cells to investigate the degree of degranulation of mast cells [24]. In this study, RBL-2H3 cells were stained with toluidine blue. The results indicated that DAP inhibited the morphological changes in RBL-2H3 cells induced by C48/80 and reduced extracellular particulate matter. The potential F-actin structure plays a material role in mast cell activation [25]. The results

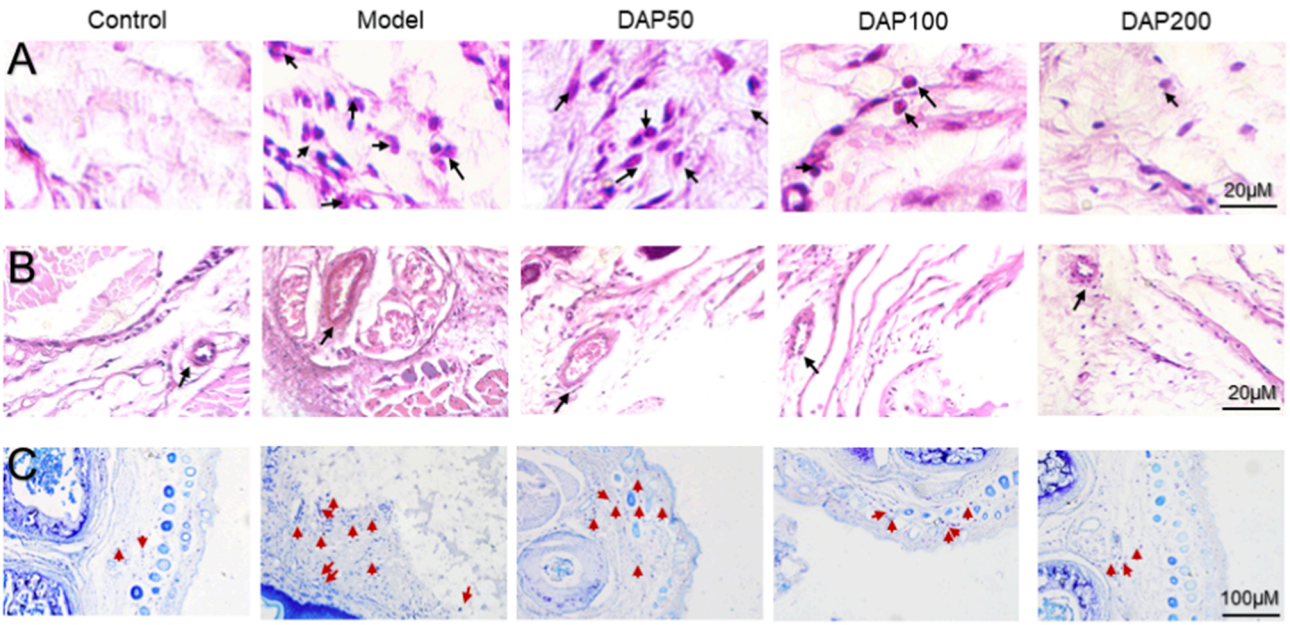

Fig. 8. DAP decreased C48/80-induced local anaphylaxis in mouse paw by inhibiting MC degranulation. The treatment group was given DAP (0, 50, 100, and 200 mg/kg) and subcutaneous injection of 50 μg/ml C48/80 into the paws. CMC-Na and subcutaneous injection of Tyrode's solution were given as controls, n = 4. A: DAP inhibits eosinophil release (H&E staining). B: DAP inhibited vasodilation (H&E staining). C: DAP inhibited mast cell degranulation (toluidine blue staining).

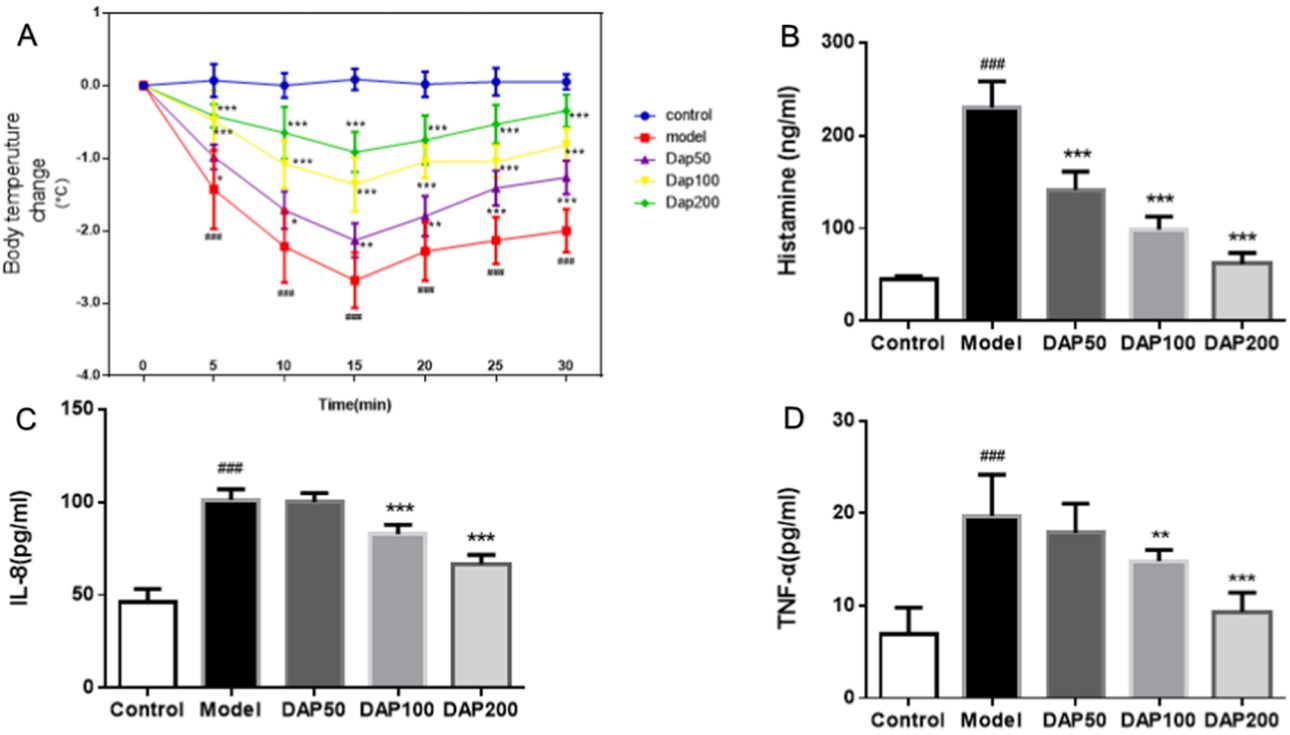

Fig. 9. DAP inhibited systemic anaphylaxis induced by C48/80 in mice. A: The change in body temperature at 30 min after injection with C48/80 was measured. B-D: Effects of DAP (0, 50, 100, and 200 mg/kg) pretreatment on serum histamine (B), IL-8 (C), and TNF-α (D) levels after C48/80 (0.3 mg/kg) injection. Data are presented as the mean ± SD, n = 6. ANOVA was used to assess the significance of statistical comparisons. (###p < 0.001vs control group ; *P < 0.05, **P < 0.01, ***P < 0.001 vs model group.).

demonstrated that DAP can inhibit the depolymerization of the F-actin structure of the cell membrane, inhibit the degranulation of RBL-2H3 cells, and inhibit the release of β-HEX, His, TNF-α and IL-8.DAP was preliminarily shown to inhibit C48/80-induced pseudo-allergy in vitro.

According to research reports, the structural expression of MRGPRX2 in skin mast cells rapidly releases histamine and inflammatory factors during degranulation so that mast cells rapidly affect tissue dynamic balance, such as increased vascular permeability. We used local skin tissue administration and tail vein injection to establish local allergic reactions and systemic allergic reactions in mice. The diameter of blue spots, paw swelling and Evans blue exudation in local allergic reactions were decreased, and the decrease in body temperature in systemic

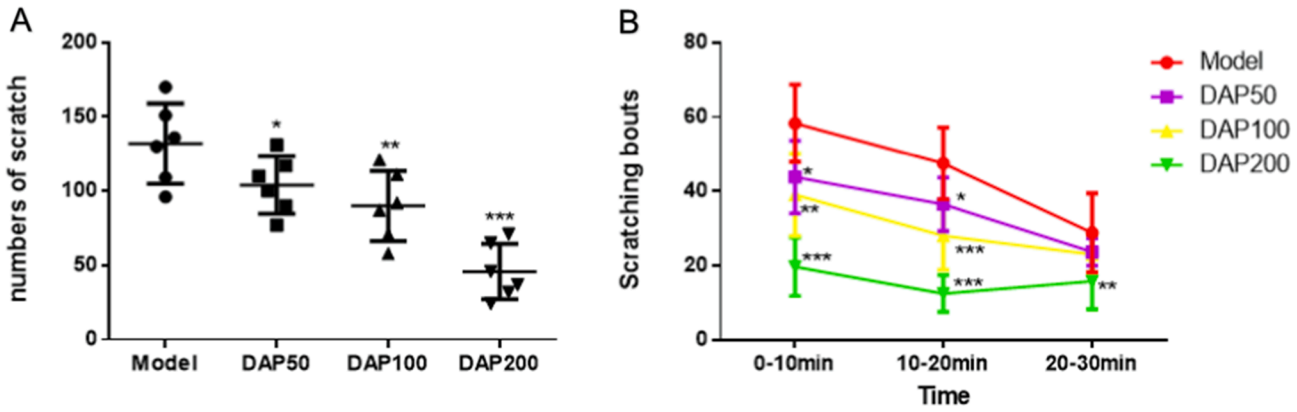

Fig. 10. DAP inhibited scratch behavior induced by C48/80 injection in mice. A: Number of scratches in 30 min. B: Scratching behavior time-course profiles at 10 min intervals. Early on in the 30-minute observation period (up to 20 min), the DAP pretreatment groups displayed a reduction in scratching episodes. Data are presented as the mean ± SD, n = 6. ANOVA was used to assess the significance of statistical comparisons. (###p < 0.001vs control group ; *P < 0.05, **P < 0.01, ***P < 0.001 vs model group.).

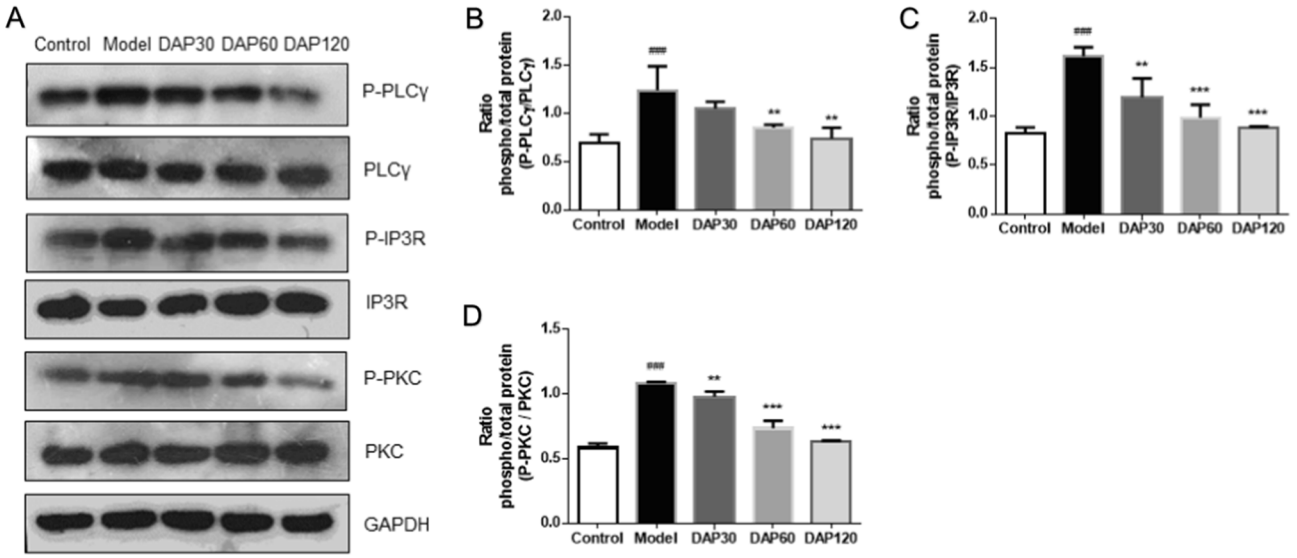

Fig. 11. DAP inhibited the expression of P-PLCγ/PLCγ, P-IP3R/IP3R and P-PKC/PKC in RBL-2H3 cells induced by C48/80. DAP downregulated the protein expression of phosphorylated PLCγ, phosphorylated IP3R and phosphorylated PKC induced by C48/80 in RBL-2H3 cells. A: Western blot analysis of the expression levels of PLCγ, IP3R, PKC Phosphorylated-PLCγ, phosphorylated-IP3 and Phosphorylated-PKC in RBL-2H3 cells treated with DAP (0, 30, 60, 120 μM) and C48/80. B-D: Quantification of PLCγ, Phosphorylated-PLCγ (B), IP3R, Phosphorylated-IP3R (C), PKC, and Phosphorylated-PKC (D) protein expression in A by ImageJ. Data are presented as the mean ± SD of three independent experiments. ANOVA was used to assess the significance of statistical comparisons. (###p < 0.001vs control group ; *P < 0.05, **P < 0.01, ***P < 0.001 vs model group.).

allergic reactions was inhibited, indicating that daphnetin can inhibit pseudo-allergy induced by C48/80. The histopathological analysis of the local allergic reaction and the inhibition of histamine and related verification factor release in the systemic allergic reaction showed that it may be related to the inhibition of mast cell activation. According to previous studies, C48/80 can activate MRGPRX2, leading to anaphylactoid itching characterized by the release of His and proteases and itching [27]. In the present study, C48/80 was injected into the back to induce itch in mice, and the number of scratches in the mice was observed. The results demonstrated that DAP significantly inhibited the number of scratches in the mice. These findings demonstrated the in vivo antipseudo-allergy effect of DAP.

According to previous reports, stimuli such as C48/80 directly trigger MRGPRX2 expression in human mast cells [28], causing PLCγ phosphorylation. PLC activation causes PIP2 hydrolysis, which yields IP3 and DAG. The “initial wave” of calcium mobilization, or the brief release of Ca^{2+} from endoplasmic reticulum storage, is induced by the interaction of IP3 and IP3R, whereas DAG binds to dormant PKC in the cytoplasm, causing PKC phosphorylation. Thus, the release of Ca^{2+} and the activation of PKC in the endoplasmic reticulum were induced [29,30]. Through MAPK-dependent pathways such as ERK1/2 and P38, persistent activation of Mrgprx2 also causes the generation of Ca^{2+}-dependent proinflammatory factors, chemokine synthesis, and mast cells [13]. In our research, DAP inhibited RBL-2H3 cell activation by decreasing the phosphorylation of PLCγ, IP3R, and PKC. ERK1/2 and P38. These results suggested that DAP may be related to the downstream signaling pathways of MRGPRX2 or MRGPRB2/B3, but the effects of daphnetin on these receptors need to be further studied.

In the present investigation, we discovered that DAP could prevent C48/80-induced pseudo-allergy. The results showed that DAP could inhibit C48/80-induced RBL-2H3 cell degranulation by inhibiting the phosphorylation of PLCγ, IP3R, PKC, p38 and ERK1/2 and could inhibit C48/80-induced local anaphylaxis, systemic anaphylaxis and itch in vivo.

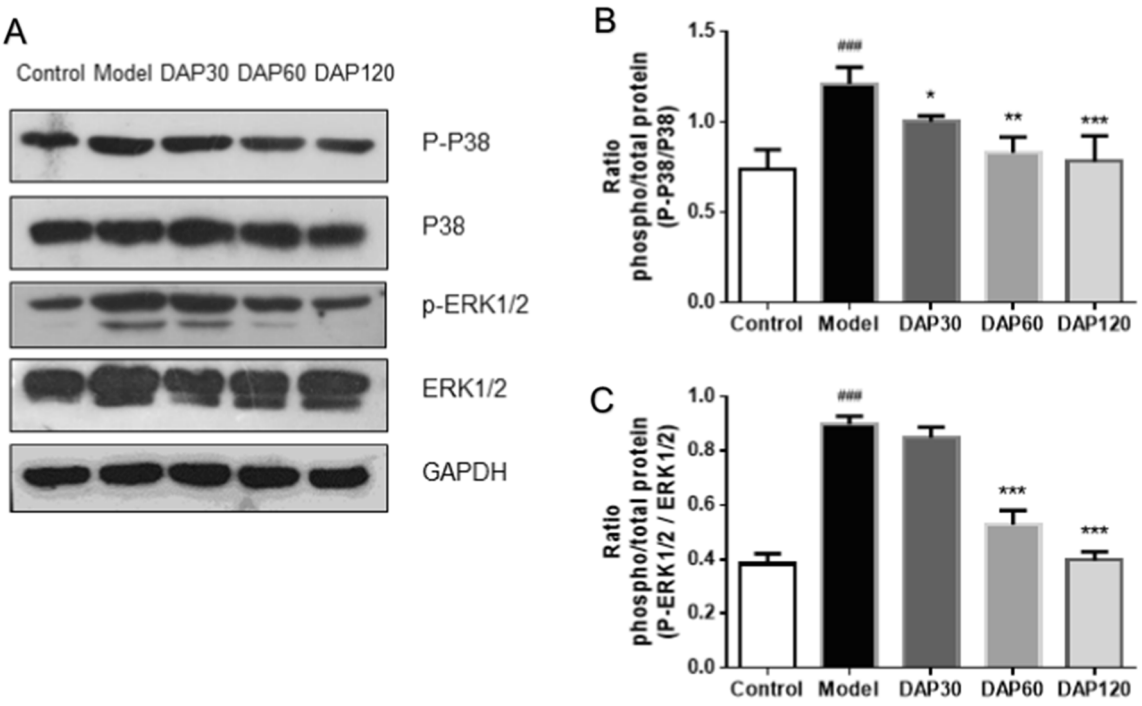

Fig. 12. DAP inhibited the expression of P-p38/p38 and P-ERK1/2 /ERK1/2 in RBL-2H3 cells induced by C48/80. DAP downregulated the protein expression of phosphorylated P38 and phosphorylated ERK1/2 induced by C48/80 in RBL-2H3 cells. A: Western blot analysis of the expression levels of P38, ERK1/2, phosphorylated P38, and phosphorylated ERK1/2 in RBL-2H3 cells treated with DAP (0, 30, 60, 120 μM) and C48/80. B-C: Quantification of P38, phosphorylated P38 (B), ERK1/2, and phosphorylated ERK1/2 (C) protein expression in A by ImageJ. Data are presented as the mean ± SD of three independent experiments. ANOVA was used to assess the significance of statistical comparisons. (###p < 0.001vs control group ; *P < 0.05, **P < 0.01, ***P < 0.001 vs model group.).

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CRediT authorship contribution statement

Jingyu Zhang: Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Ling Hong:** Investigation, Validation. **Ping Zhang:** Methodology, Investigation. **Yanjie Wang:** Resources, Software. **Tie Hong:** Funding acquisition, Supervision, Project administration, Writing – review & editing.

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.

Data availability

No data was used for the research described in the article.

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