

Pterostilbene Inhibits FcεRI Signaling through Activation of the LKB1/AMPK Pathway in Allergic Response

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Cite This: *J. Agric. Food Chem.* 2020, 68, 3456–3465



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ABSTRACT: In this study, the role and mechanism of pterostilbene (Pts) in mast cell degranulation in vitro and in vivo were investigated. The results showed that Pts inhibited mast cell-mediated local passive allergic reactions in mice. In addition, treatment with Pts reduced both histamine release and calcium influx in rat peritoneal mast cells and RBL-2H3 cells and reduced IgE-mediated mast cell activation. Furthermore, the mechanism underlying Pts inhibition of mast cell signaling was probed via studying the effects of Pts on liver kinase B1 (LKB1), including the use of the LKB1 activator metformin and siRNA knockdown of LKB1. The data showed that Pts reduced the release of inflammatory mediators such as tumor necrosis factor- α , interleukin-6, leukotriene C4, and prostaglandin D2 in mast cells by activating the LKB1/adenosine monophosphate (AMP)-activated protein kinase (AMPK) signaling pathway. Furthermore, Pts inhibited phosphorylation of FcεRI and FcεRI-mediated degranulation in RBL-2H3 cells. These effects were attenuated after LKB1 knockdown. Taken together, Pts could inhibit FcεRI signaling through activation of the LKB1/AMPK signaling pathway in IgE-mediated mast cell activation. Thus, Pts might be an effective therapeutic agent for mast cell-mediated allergic diseases.

KEYWORDS: pterostilbene, mast cell degranulation, LKB1, AMPK, FcεRI signaling

INTRODUCTION

Allergic reactions can occur in all organs and tissues. Mast cells are the main effector cells of allergic reactions and can trigger inflammatory reactions associated with asthma, atopic dermatitis, and sinusitis.¹ Mast cells are widely found in tissues with adequate blood supply, especially in areas where tissue surfaces are often in contact with outside air, such as skin tissue, airway tissue, gastrointestinal tissue, and colon tissue.² When the host is again stimulated by the allergen, mast cells can degranulate and release inflammatory factors, including pro-inflammatory cytokines and chemokines, which mediate the development of type I hypersensitivity. Among them, histamine is the main cause of vasodilation, increased vascular permeability, edema, hypothermia, and leukocyte aggregation.^{3,4} In the advanced stage of allergic inflammation, mast cells release chemotactic and pro-inflammatory mediators, such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, prostaglandin D2 (PGD2), and leukotriene C4 (LTC4).⁵ Activation of mast cells induces FcεRI signaling, which in turn leads to activation of mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt), and I κ B kinase (IKK)-nuclear factor- κ B (NF- κ B) pathways.^{6,7} These pathways all play an important role in the induction of chemokines and cytokines.^{6,7}

Liver kinase B1 (LKB1) is well known as a tumor suppressor enzyme. Meanwhile, it also acts as the main kinase to induce adenosine monophosphate (AMP)-activated protein kinase (AMPK) phosphorylation.^{8,9} AMPK is a core detector which can regulate the metabolism of substances and energy and promote cell homeostasis. It coordinates the balance of energy

supply and demand in cells by sensing various stimuli inside and outside cells.^{10,11} Metformin, the first-line drug of type 2 diabetes mellitus, inhibits many downstream signaling pathways of AMPK.¹² Metformin is also considered as an AMPK activator.¹³ It is reported that metformin modulates the activation of mast cells by suppressing FcεRI and aryl hydrocarbon receptor-mediated signaling or their combination.¹⁴ Furthermore, metformin suppresses LPS-induced inflammatory responses and ameliorates allergic contact dermatitis.¹⁵ In addition, studies have shown that activation of the LKB1/AMPK/mTOR signaling pathway can inhibit nonsmall cell lung cancer⁸ and play an important role in maintaining oxygen supply in large blood vessels such as the heart, brain, and kidney.⁹ In recent years, the LKB1/AMPK signaling pathway has been found to play an important role in allergic diseases. For example, Hwang et al. found that LKB1 and its downstream kinase AMPK were involved in the negative regulation of FcεRI-caused mast cell activation.¹⁶ Thus, the LKB1/AMPK signaling pathway may become an important target of anti-allergic treatment.^{5,17}

Resveratrol is a polyphenolic substance with many protective properties. It can not only protect nerves but also inhibit cancer.¹⁸ Resveratrol is also an agonist of Sirt1, which can

Received: November 20, 2019

Revised: February 14, 2020

Accepted: February 25, 2020

Published: February 25, 2020

activate Sirt1 and its downstream signaling pathways,^{19,20} including LKB1/AMPK signaling.^{21,22} Pterostilbene (Pts) is a derivative of resveratrol. It is found in many berries, and its oral absorption and bioavailability are better than those of resveratrol. Studies have confirmed that Pts has anti-inflammatory,²³ anti-oxidant,²⁴ anti-allergic,²⁵ and liver protection effects,²⁶ can regulate blood lipids,²⁷ and has a better prevention and treatment effect for neurological diseases and cancer.²⁸ Furthermore, it is reported that LKB1/AMPK activation by Pts contributes to mitochondrial superoxide-free radical reduction, endothelial cell senescence, and increase of the mRNA expression of several genes related to mitochondrial function.²⁹ Pts reduces liver injury by activating the Nrf2 anti-oxidative defense system via the AMPK/Akt/GSK3 β pathway.²⁶ Pts can also affect calcium ions in the body. It is reported that Pts at a concentration greater than 10 μ M could suppress type-1 Ca²⁺ and transient outward K⁺ currents in GH3 cells.³⁰

Herein, we investigated the effects of Pts on mast cell degranulation in vitro and in vivo and explored how Pts alleviated allergic diseases. Our findings may provide insights into the pathophysiology of allergic diseases.

MATERIALS AND METHODS

Animals. Male BALB/c mice (6 week old, 20 $\pm$ 2 mg, n = 40) and male Sprague-Dawley rats (220 $\pm$ 20 g, n = 20) were purchased from the Medical Department of Yanbian University (Yanji, China). The mice and the rats were kept under standard conditions. All animal experiments were approved by the Animal Protection Committee of Yanbian University (SCXK (JL)2017-0003).

Passive Cutaneous Anaphylaxis Test. Mice were divided into normal group, IgE + Ag group, Pts low dose group (10 mg/kg), and Pts high dose group (20 mg/kg), with 10 mice in each group. The mice in Pts groups were given 10 and 20 mg/kg Pts by gavage once daily for 4 weeks. The mice in the IgE + Ag group and in Pts groups were sensitized by subcutaneous injection of 0.2 μ g of anti-DNP IgE in 20 μ L of phosphate-buffered saline after 24 h of the last gavage, and 1 day later, the mice were given the DNP-HSA (1 mg) and 1% Evans blue (1:1) mixture by tail vein injection. The mice in the normal group were given an equal amount of saline. At 30 min after the challenge, mild anesthesia was induced in mice and a digital micrometer (Mitutoyo, no. 7326, Japan) was used to measure the thickness of the ear. Then, mice were sacrificed under deep anesthesia. The ears were collected. After incubating the removed skin in formamide for 24 h at 55 $^{\circ}$ C, the extravasated Evans blue dye was extracted. The amount of dye absorbance was measurement at 620 nm wavelength with a Spectra MAX PLUS spectrophotometer (Molecular Devices, CA, U.S.A.).

Transmission Electron Microscope. Normal ear tissue was immobilized in 2.5% glutaraldehyde for 24 h and then immobilized in 2% osmium acid for 2 h, dehydrated in gradient propionic acid, and immersed in the epoxy resin and acetone mixture (1:1) for 24 h before embedding. Tissue sections were stained with uranium acetate and lead nitrate and then observed and photographed by transmission electron microscopy (Jeol JEM-1230, Japan).

Histology Analysis. After fixation and embedding in paraffin, the ears were cut into sections (5 μ m). Hematoxylin & eosin and toluidine blue staining were performed according to the routine procedure.^{31,32}

Rat Peritoneal Mast Cell Preparation. To obtain rat peritoneal mast cells (RPMCs), rats were anesthetized with ether and intraperitoneally injected with 10 mL of HERMES-Tyrod buffer (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO₃, 2.7 mM KCl, 0.3 mM NaH₂PO₄, and 0.1% gelatin). Abdominal lavage fluid was collected after 90 s of mild abdominal massage. The collected mast cells were purified using Percoll (Pharmacia, Uppsala, Sweden).³³ The RPMC preparations were about 95% pure as assessed by toluidine

blue staining, and more than 98% of the cells were viable as judged by trypan blue uptake. Cells were resuspended in HEPES-Tyrod resuspended buffer.

Cell Culture and Treatment. RBL-2H3 cells purchased from Shanghai Cytobiology Research institute (Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, New York) with 10% fetal bovine serum (FBS, Gibco, Grand Island, New York), 100 U/mL penicillin G, and 100 μ g/mL streptomycin and cultured at 37 $^{\circ}$ C, 5% CO₂. For treatment, 10 μ g/mL anti-DNP IgE (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was added to RBL-2H3 cells or RPMCs for 6 h. Then, cells were activated with DNP-HSA (Sigma) (100 ng/mL) for 30 min. Two concentrations of Pts (15 and 30 μ M; Figure 1A; with purity $\geq$ 97%; Sigma) or metformin (50 μ M;³⁴ Abcam, Cambridge, MA, USA) were added into the cells and incubated for 30 min at 37 $^{\circ}$ C before challenging with DNP-HSA.

MTT Assay. After adding Pts of different concentrations (0, 1, 10, 50, and 100 μ M) to RBL-2H3 cells and RPMCs, the cells were incubated in a 37 $^{\circ}$ C CO₂ incubator for 24 h. MTT (Sigma-Aldrich, St. Louis, MO, USA) was added and further incubated for 4 h. After the purple crystals were precipitated, DMSO was added and the absorbable absorbance at 570 nm was measured using a

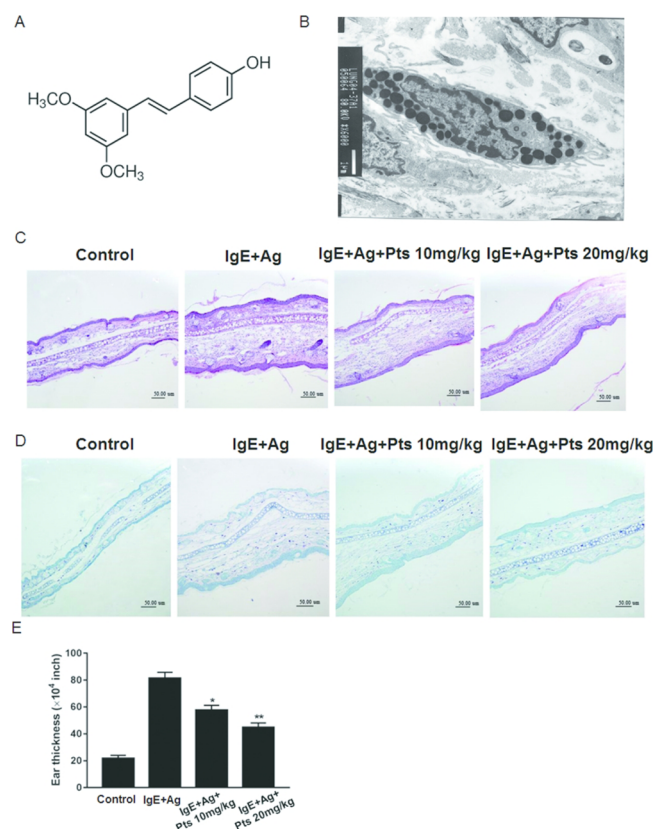


Figure 1. Pts suppresses IgE/Ag-induced mast cell activation. (A) Chemical structure of Pts. (B) Normal ear tissue was analyzed under an electron microscope. Magnification was 10,000 $\times$. (C,D) Representative photomicrographs of ear sections stained with hematoxylin & eosin and toluidine blue (magnification was 200 $\times$). Scale bars indicate 50 μ m. Sampling was performed 30 min after the last challenge in mice. (E) Ear thickness measured with a dial thickness gauge. Control, normal mice; IgE + Ag, mice sensitized with anti-DNP IgE for 6 h and challenged with DNP-HSA for 30 min. Pts was administered by gavage at doses of 10 and 20 mg/kg once daily for 4 weeks. (F) Amount of Evans blue and inhibition rate was presented. Data were shown as means $\pm$ SEM (n = 10). * P < 0.05 and ** P < 0.01 vs the IgE + Ag group.

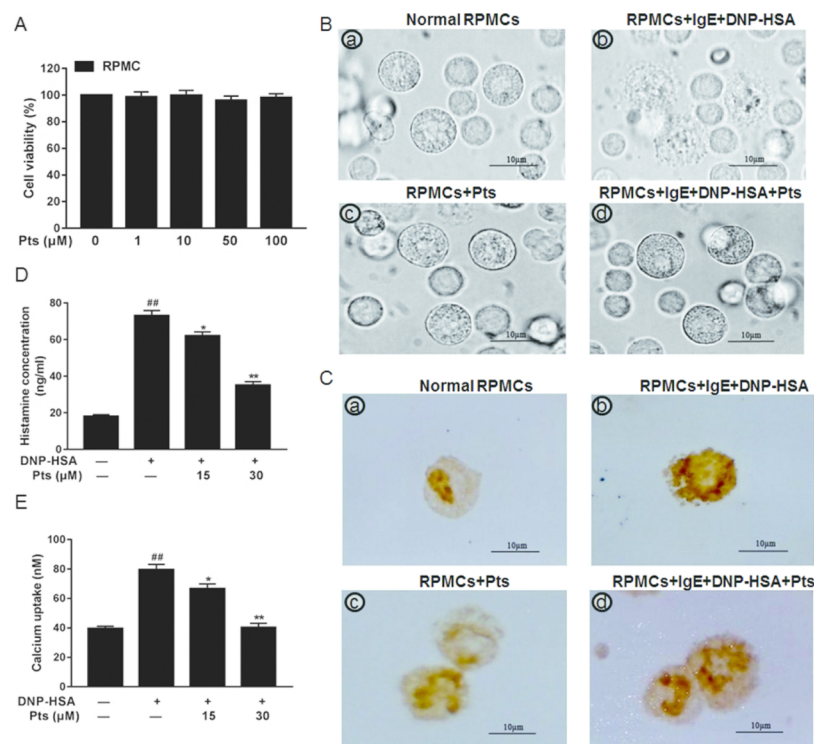


Figure 2. Effect of Pts on cell viability and degranulation of IgE/Ag-induced RPMCs. (A) Cell viability represented by relative absorbance compared to the control. RPMCs were treated with various concentrations of Pts. (B) Effect of Pts on anti-DNP IgE-mediated degranulation of RPMCs. Scale bar: 10 μ M. (a) Normal RPMCs in HEPES-Tyrod buffer; (b) RPMCs were sensitized with 10 μ g/mL anti-DNP IgE for 6 h and challenged with 100 ng/mL DNP-HSA; (c) RPMC incubation with 30 μ M Pts at 37 $^{\circ}$ C for 30 min; and (d) prior to the challenge with 100 ng/mL DNP-HSA, cells were preincubated with 30 μ M Pts at 37 $^{\circ}$ C for 30 min. (C) Immunohistochemical staining was performed to assess the distribution of IL-6. Scale bar: 10 μ M. (a) Normal RPMCs in HEPES-Tyrod buffer; (b) RPMCs were sensitized with 10 μ g/mL anti-DNP IgE for 6 h and challenged with 100 ng/mL DNP-HSA; (c) RPMC incubation with 30 μ M Pts at 37 $^{\circ}$ C for 30 min; and (d) prior to the challenge with 100 ng/mL DNP-HSA, cells were preincubated with 30 μ M Pts at 37 $^{\circ}$ C for 30 min. (D) Histamine release and (E) calcium uptake by RPMCs sensitized with 10 μ g/mL anti-DNP IgE for 6 h and preincubated with Pts (15 and 30 μ M) at 37 $^{\circ}$ C for 30 min prior to challenge with DNP-HSA (100 ng/mL) for 30 min. Data were shown as means $\pm$ SEM. of three independent experiments. ^{##} P < 0.01 vs untreated cells, ^{*} P < 0.05 and ^{**} P < 0.01 vs the cells stimulated with IgE and Ag.

spectrophotometer (Molecular Devices, Sunnyvale, Spectra MAX PLUS, CA, USA).

Histamine Content Measurement. The radio enzymatic method was used to detect histamine release by RPMCs and RBL-2H3 cells. Briefly, cells were sensitized with anti-DNP IgE, treated with Pts and metformin, and then challenged with DNP-HSA for 30 min. The supernatant was collected by centrifugation at 4 $^{\circ}$ C for 10 min. Then, the concentration of histamine was measured by the radio enzymatic method.³⁵

⁴⁵Ca Uptake Measurement. As for calcium uptake of mast cells, RPMCs and RBL-2H3 cells were resuspended in HEPES-Tyrod buffer containing ⁴⁵Ca (1.5 mCi/mL, PerkinElmer Life Sciences, MA, USA) at 4 $^{\circ}$ C for 10 min. Then, the cells were sensitized with anti-DNP IgE, treated with Pts and metformin, and then challenged with DNP-HSA for 30 min. After centrifugation, 10% Triton X-100 was added for cell lysis. Radioactivity was measured with a liquid scintillation analyzer (Canberra Industries, Inc., Meriden, CT, USA).

Immunohistochemistry. The treated RPMCs were incubated with primary antibodies against IL-6 (Abcam, ab9324, Cambridge, MA, USA) followed by the incubation with peroxidase-conjugated secondary antibodies (Goat anti-mouse IgG-HRP, Abcam, ab205719). After immunostaining, the slides were counterstained with hematoxylin, mounted (ThermoShandon, Pittsburgh, PA, USA), and observed under a microscope.

Enzyme-Linked Immunosorbent Assay. The release of inflammatory mediators, including TNF- α , IL-6, LTC₄, and PGD₂, was measured with enzyme-linked immunosorbent assay (ELISA). ELISA kits were purchased from Santa Cruz Biotechnology and Cayman Chemicals. ELISA was performed on a 96-well Nunc

immune plate according to the manufacturer's protocol. For mast cell stimulation, RBL-2H3 cells were sensitized with anti-DNP IgE for 6 h and then stimulated with DNP-HSA for 30 min at 37 $^{\circ}$ C. Pts or metformin was added 30 min prior to the addition of DNP-HSA. The absorbance intensity was detected using a spectrophotometer at a wavelength of 450 nm.

Western Blot. Total proteins were extracted from cells. Protein concentration was determined using a BCA protein assay kit (Beyotime Biotechnology, China). The protein sample was separated and transferred to the nitrocellulose membrane. After blocking, primary antibodies were added and incubated at 4 $^{\circ}$ C overnight. The antibodies against phosphorylated forms of LKB1 (Ser428; #3482), AMPK α (Thr172; #2535), ACC (Ser79; #11818), PLC γ 1 (Tyr783; #2821), Akt (Ser473; #4060), p38 (Thr180/Tyr182; #4631), ERK1/2 (Thr202/Tyr204; #4376), JNK (Thr183/Tyr185; #4668), IKK (Ser176/180; #2697), and mTOR (Ser2448; #2971) and the antibodies against LKB1 (#3047), AMPK α (#5831), ACC (#3676), Akt (#4691), p38 (#8690), ERK1/2 (#4695), JNK (#9252), and mTOR (#2983) were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibodies against PLC γ 1 (sc-81) and IKK (sc-7607) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit IgG-HRP (Cell Signaling Technology) secondary antibodies were used. Amersham Imager 600 (Bio-TEK, Vermont, GE, USA) was used for image acquisition.

Transfection of siRNA. RBL-2H3 cells were transfected with LKB1 siRNA and control siRNA, which were both purchased from Invitrogen (Carlsbad, CA, USA). The cells were transfected according to the instructions of Lipofectamine 2000. After 48 h of transfection,

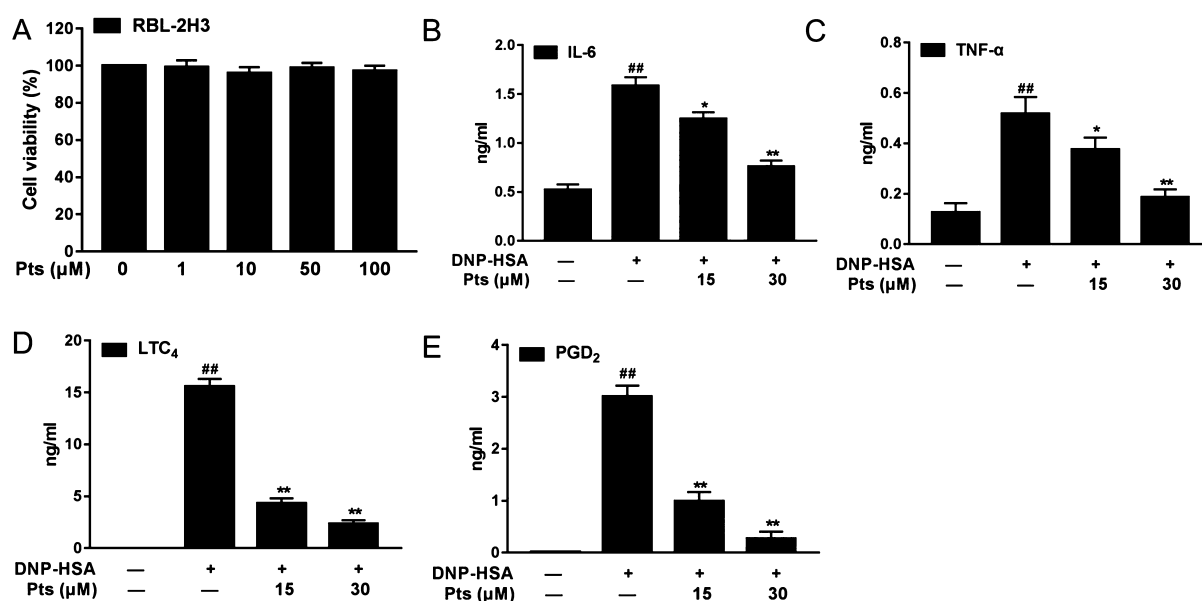


Figure 3. Effect of Pts on cell viability and inflammatory mediators of IgE/Ag-induced RBL-2H3 cells. (A) Cell viability represented by relative absorbance compared to the control. RBL-2H3 cells were treated with various concentrations of Pts. IgE-induced release of IL-6 (B), TNF- α (C), LTC₄ (D), and PGD₂ (E) was evaluated by ELISA. Data were shown as means $\pm$ SEM. of three independent experiments. ## P < 0.01 vs untreated cells, * P < 0.05 and ** P < 0.01 vs the cells stimulated with IgE and Ag.

the cells were sensitized with DNP-IgE for 6 h and treated with Pts for 30 min at 37 °C and then challenged with DNP-HSA for 30 min.

Statistical Analysis. All results were analyzed using SPSS 17.0 (IBM, Armonk, NY, USA). The experimental data were expressed as mean $\pm$ SEM. All the histograms were plotted using Prism 7.0 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance followed by Tukey's post hoc analysis was used. A P value < 0.05 was considered statistically significant.

RESULTS

Pts Reduces Anti-DNP IgE-Induced Passive Cutaneous Anaphylaxis Response in Mice. A normal-sized mast cell from the ear tissue is shown in Figure 1B. There were high electron density particles in the cytoplasm, no swelling in the cytoplasm, no mutual fusion between the particles, and no obvious vacuolar formation. BALB/c mice were used for the evaluation of the effect of Pts on IgE/Ag-reduced passive cutaneous anaphylaxis (PCA) in vivo. To determine the status of mast cells, ear sections were stained with hematoxylin & eosin and toluidine blue (Figure 1C,D). The results showed that the ear thickness was increased by injection of antigen and decreased by administering Pts (Figure 1E). Moreover, the amount of Evans blue was decreased by administering Pts (Table S1). These results suggest that Pts inhibits the activation of mast cells and thus decreases anti-DNP IgE-mediated PCA response in vivo.

Pts Reduces Degranulation, Histamine Release, and Intracellular Calcium Level of RPMCs. The effect of Pts on cell viability was examined by MTT assay. RPMCs were incubated with different concentrations (1–100 μ M) of Pts for 24 h. The results showed that Pts up to 100 μ M did not significantly affect the viability of RPMCs (P > 0.05) (Figure 2A). However, IgE-mediated degranulation of RPMCs treated with Pts was obviously inhibited (Figure 2B). Immunohistochemical staining results showed that IL-6 expression was obviously higher in IgE-mediated RPMCs than that in untreated cells (Figure 2C), indicating that Pts treatment could reduce IL-6. In addition, Pts also inhibited histamine

release induced by DNP IgE in RPMC cells (P < 0.05) (Figure 2D). To explore the potential mechanisms, we measured the intracellular calcium levels. The results showed that after exposure to DNP-HSA, the intracellular calcium level of RPMCs was significantly higher than that of control group cells, while Pts inhibited the elevation of the intracellular calcium level (P < 0.05) (Figure 2E). These results indicate that Pts could decrease the degranulation, histamine release, and intracellular calcium level of mast cells.

Pts Decreases Inflammatory Cytokines in RBL-2H3 Cells. We also detected the effect of Pts on RBL-2H3 cell viability by MTT. The results showed that different concentrations of Pts had no significant effect on the activity of RBL-2H3 cells (P > 0.05) (Figure 3A). As mast cell-derived cytokines play an important biological role in inducing delayed-type hypersensitive allergic and inflammatory responses, we then detected inflammatory cytokines of IL-6, TNF- α , LTC₄, and PGD₂ in RBL-2H3 cells by ELISA. Again, the results showed that Pts inhibited the cytokine increase under the activation of mast cells (P < 0.05) (Figure 3B–E).

Pts Activates the LKB1/AMPK Axis and Inhibits Fc ϵ RI-Induced Activation in RBL-2H3 Cells. It has been reported that Pts could activate the AMPK signal in different diseases.^{26,36,37} Thus, we investigated the effect of Pts on the phosphorylation of signaling mediators in the LKB1-AMPK pathway and on Fc ϵ RI-mediated signaling in RBL-2H3 cells. After sensitizing RBL-2H3 cells for 6 h, RBL-2H3 cells were treated with Pts (15 and 30 μ M) for 1 h. The phosphorylated LKB1 and AMPK and the level of total LKB1 and AMPK and their downstream signaling pathway proteins in mast cells were detected by western blot. As shown in Figure 4, the phosphorylation of LKB1, AMPK, and ACC was increased after Pts treatment, indicating that Pts could enhance the activity of LKB1, AMPK, and ACC in RBL-2H3 cells. In addition, the western blot results of proteins involved in Fc ϵ RI-mediated signaling showed that Pts could significantly reduce the phosphorylation of PLC γ 1 and MAPK pathways, including

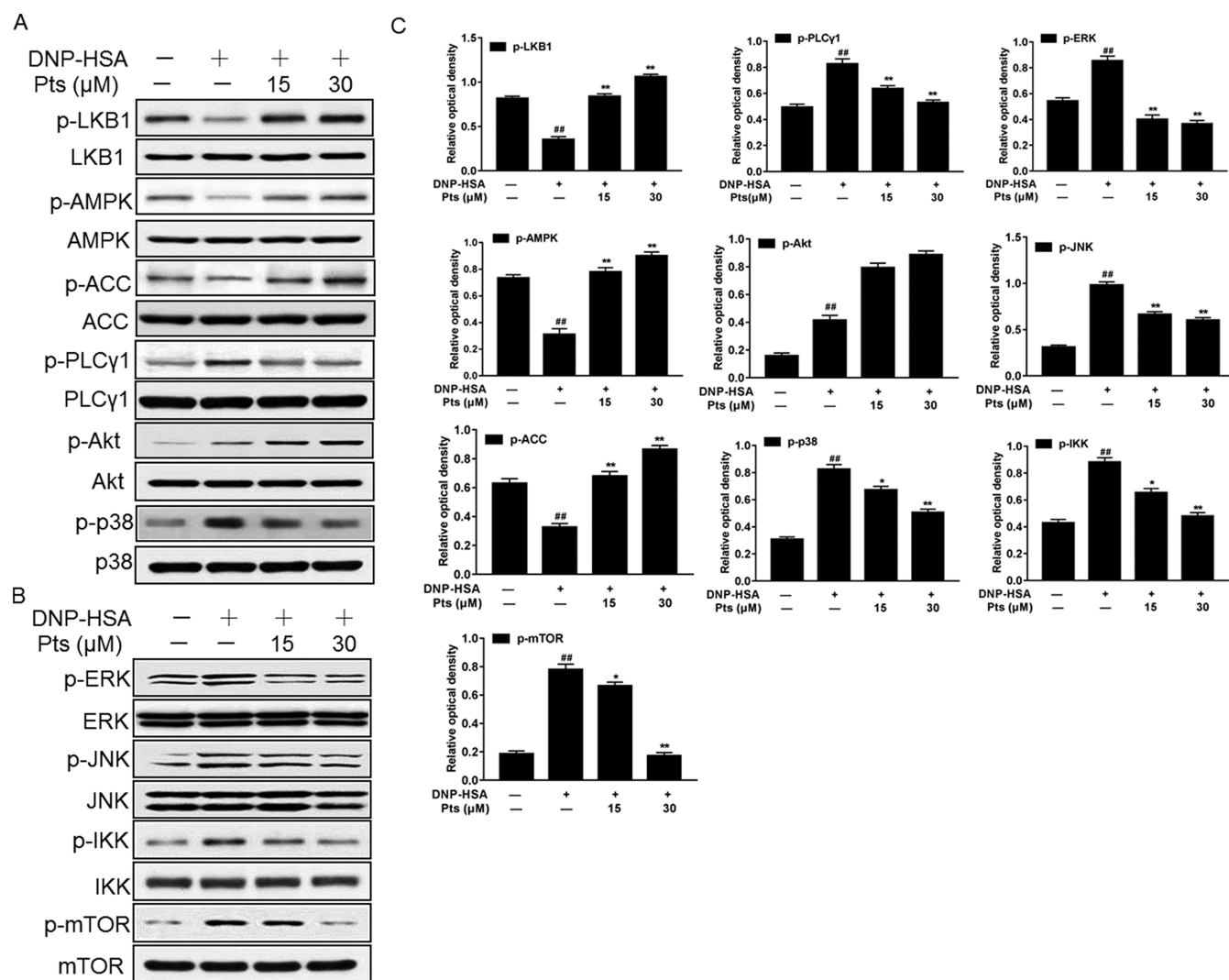


Figure 4. Pts activates the LKB1/AMPK signaling pathway in IgE/Ag-induced RBL-2H3 cells. (A,B) RBL-2H3 cells were sensitized with anti-DNP IgE for 6 h, incubated with the indicated concentrations of Pts for 30 min, and then stimulated for 30 min with DNP-HSA. Protein levels were detected with western blot. (C) Band intensities of these proteins were expressed as a ratio to those of total proteins. Data were shown as means $\pm$ SEM. of three independent experiments. ^{##} $P < 0.01$ vs untreated cells, ^{*} $P < 0.05$ and ^{**} $P < 0.01$ vs the cells stimulated with IgE and Ag.

p38, ERK, and JNK, and phosphorylation of IKK and mTOR. In this process, we found an interesting result that Pts increased the phosphorylation of Akt. All the abovementioned results showed that Pts not only activated the LKB1/AMPK pathway but also inhibited FcεRI signaling.

Activation of the LKB1 Signal Reduces the Degranulation of Mast Cells. Metformin is an agonist of LKB1-AMPK. We found that metformin and Pts reduced histamine release and intracellular calcium levels in mast cells (Figure 5A,B). These results suggest that activation of the LKB1/AMPK signaling pathway could effectively alleviate the degranulation effect of mast cells. Meanwhile, we also found that the activation of LKB1/AMPK could reduce the levels of IL-6 (Figure 5C), TNF- α (Figure 5D), LTC4 (Figure 5E), and PGD2 (Figure 5F) in the DNP-HSA-stimulated mast cell. This indicates that activation of the LKB1/AMPK signaling pathway could reduce the secretion of inflammatory cytokines in the activated mast cells. In addition, we found that the activation of LKB1/AMPK not only increased phosphorylation of LKB1, AMPK, and ACC but also decreased phosphorylation of PLCγ1, p38, ERK, JNK, IKK, and mTOR (Figure 5G–I).

Moreover, consistent with our previous findings, the activation of the LKB1/AMPK pathway with metformin and Pts caused a transient increase of Akt phosphorylation. To a certain extent, activation of the LKB1-AMPK signaling pathway may lead to inhibition of phosphorylation of the MAPK signal, IKK, and mTOR and a temporary increase of Akt phosphorylation.

Effect of Knockdown of LKB1 on IgE/Ag-Stimulated Mast Cell Activation. To further verify that Pts inhibits mast cell activation by activating the LKB1/AMPK pathway and the relationship between the LKB1/AMPK signaling pathway and the FcεRI signal during mast cell degranulation, we silenced LKB1 signaling by siRNA (Figure 6). The results showed that the knockdown of LKB1 increased the release of histamine in mast cells (Figure 6A), increased the influx of Ca²⁺ (Figure 6B), and increases the levels of inflammatory mediators such as IL-6 (Figure 6C), TNF- α (Figure 6D), LTC4 (Figure 6E), and PGD2 (Figure 6F). By western blot, we also found that LKB1 knockdown increased FcεRI-induced phosphorylation of PLCγ1, p38, ERK, JNK, IKK, and mTOR, but unexpectedly decreased the phosphorylation of Akt (Figure 6G,H). However, the effect of Pts on mast cell activation, release of

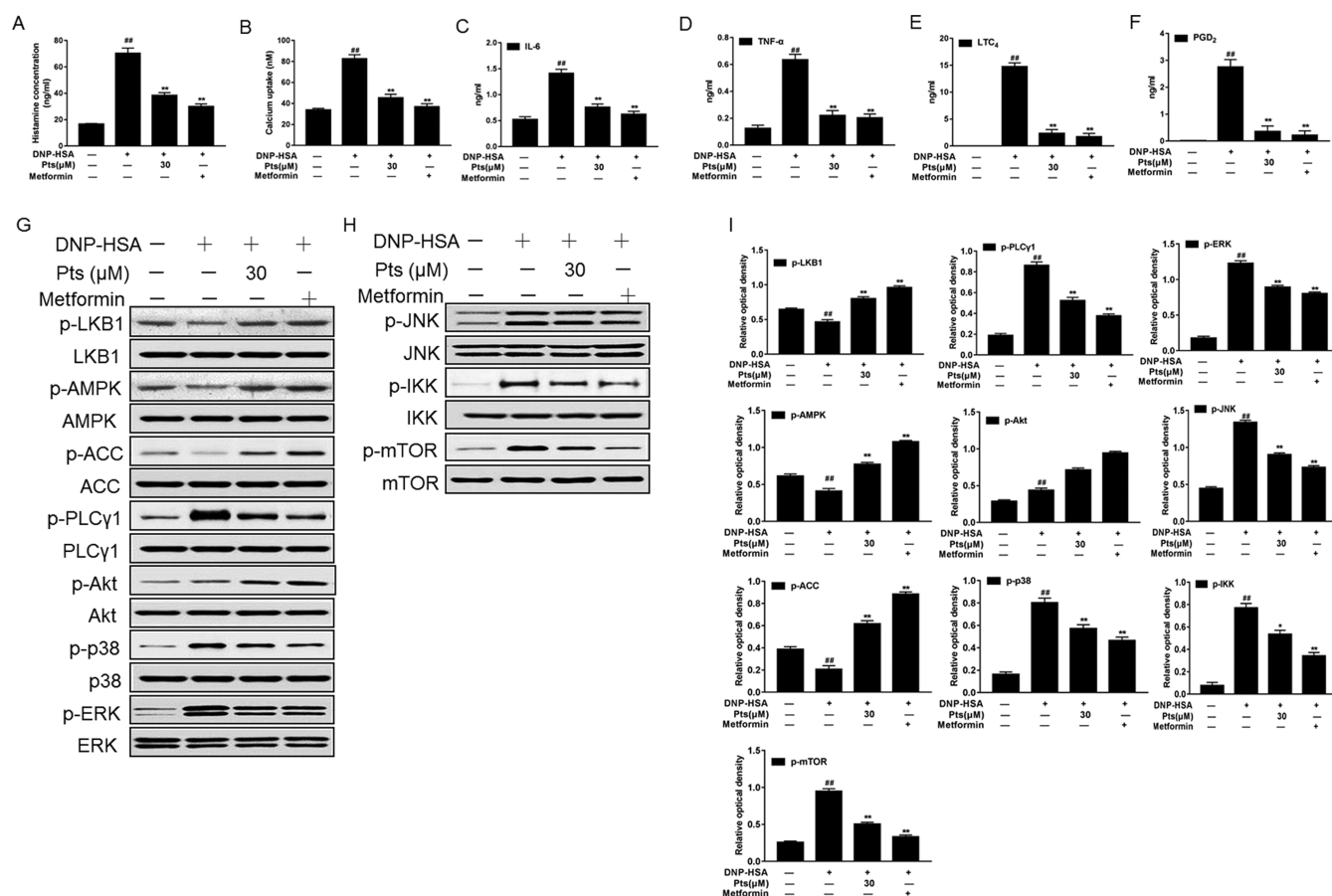


Figure 5. Activation of LKB1/AMPK results in FcεRI inhibition in IgE/Ag-induced RBL-2H3 cells. (A) Histamine release and (B) calcium uptake by RBL-2H3 cells sensitized with 10 μg/mL anti-DNP IgE for 6 h and pre-incubated with Pts (30 μM) or metformin (50 μM) at 37 °C for 30 min prior to challenge with DNP-HSA (100 ng/mL). IgE-induced release of IL-6 (C), TNF-α (D), LTC4 (E), and PGD2 (F) were evaluated in RBL-2H3 cells. (G,H) Protein levels were detected with western blot. (I) Band intensities of these proteins were expressed as a ratio to those of total proteins. Data were shown as means ± SEM. of three independent experiments. ##*P* < 0.01 vs untreated cells, **P* < 0.05 and ***P* < 0.01 vs the cells stimulated with IgE and Ag.

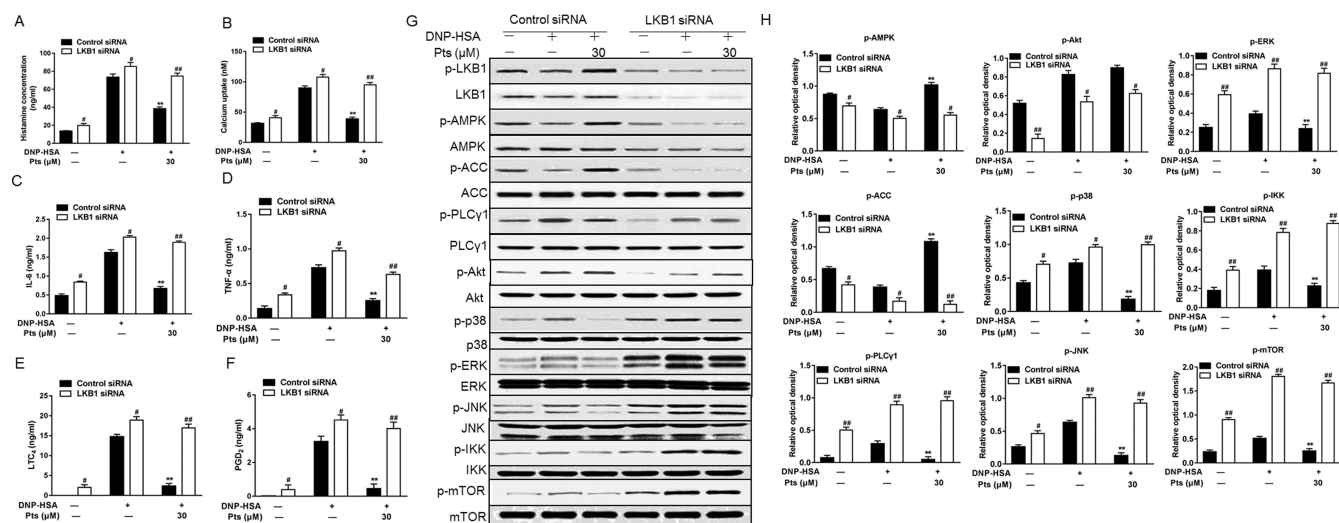


Figure 6. Knockdown of LKB1 abolishes the anti-allergic effect of Pts on RBL-2H3 cells. RBL-2H3 cells were treated with siRNA for LKB1 for 48 h. (A) Histamine release and (B) calcium uptake by RBL-2H3 cells sensitized with anti-DNP IgE for 6 h, incubated with 30 μM Pts for 30 min, and simulated with DNP-HSA for 30 min. Ag-induced release of IL-6 (C), TNF-α (D), LTC4 (E), and PGD2 (F) were evaluated in RBL-2H3 cells. (G) Protein levels were detected with western blot. (H) Band intensities of these proteins were expressed as a ratio to those of total proteins. Data were shown as means ± SEM. of three independent experiments. #*P* < 0.05 and ##*P* < 0.01 vs control siRNA in each group, ***P* < 0.01 vs DNP-HSA alone in each group.

inflammatory mediators, and phosphorylation of proteins was abolished after knockdown of LKB1. These results demonstrate that Pts inhibits the activation of mast cells by activating LKB1/AMPK signaling to block FcεRI signaling.

DISCUSSION

Type I hypersensitivity is a multisystem allergic reaction involving a variety of immune cells, cytokines, and inflamma-

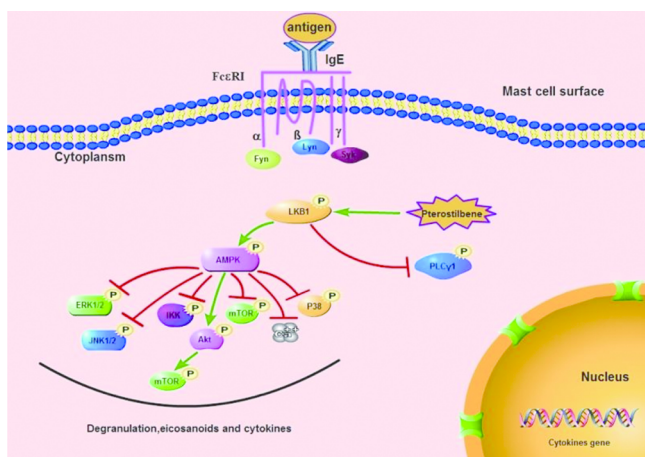


Figure 7. Schematic illustration of the mechanisms of the inhibitory effect of Pts on the FcεRI signaling pathway. Pts suppresses FcεRI signaling pathway activation in mast cells by activating LKB1/AMPK, subsequently inhibiting the pro-inflammatory cytokine secretion.

tory mediators. The binding of IgE to the high-affinity FcεRI receptor on the surface of target cells is the key to type I hypersensitivity.³⁸ The mast cell surface, which is the main effector cell involved in type I hypersensitivity, has a high level of FcεRI.¹ When the allergen enters the body for the first time, it induces the cells to produce the IgE antibody and then the FcεRI receptor on mast cells binds the IgE antibody to sensitize the body. When the same allergen re-invades the host, the complex of IgE and the allergen cross-links with the FcεRI receptor, leading to phosphorylation of Tyr upstream of FcεRI receptor signaling,^{39,40} which further leads to a large influx of Ca²⁺ and histamine release. In turn, this will induce mast cell activation and degranulation to release a large number of inflammatory factors, which play an important role in the occurrence of allergic diseases.⁴

Pts is a natural polyphenolic compound found mainly in blueberries, grapes, and other berries. As a derivative of resveratrol, Pts also has anti-inflammatory, anti-oxidant, and neuroprotective effects.^{23,24,41,42} Moreover, compared with resveratrol, Pts has stronger lipophilicity and bioavailability. Studies have reported that IgE/Ag-induced activation of mast cells is negatively regulated by the LKB1-AMPK pathway and thus that agonists of the LKB1-AMPK pathway might alleviate symptoms of allergic diseases.^{5,43} Furthermore, Kosuru et al. found that the cardioprotective effects of Pts against myocardial ischemia acted through activation of AMPK.³⁷ To explore whether Pts can inhibit mast cell activation and its possible mechanism, we therefore tested the anti-allergic effects of Pts using in vitro and in vivo models. In the animal model, Pts reduced the PCA response and ear thickness of mice induced by IgE/Ag. At the same time, the amount of Evans blue was decreased by Pts. These results indicate that

Pts suppresses the immediate-type allergic reaction through the inhibition of mast cell degranulation.

Histamine plays an important role in the regulation of allergy and inflammation. It can cause telangiectasias, increased vascular permeability, and edema. Histamine is also positively related to the severity of allergic reaction.^{44,45} Therefore, histamine can be used as a marker of mast cell activation. Calcium is a crucial secondary messenger in mast cell signaling, and it is significant to regulate histamine release by mast cells.^{46,47} In this study, we measured the levels of histamine and intracellular calcium in RPMC and RBL-2H3 cells. Pts reduced histamine levels in mast cells and induced an attenuation of intracellular calcium in mast cells. These results suggest that Pts could reduce the histamine level and block mast cell activation through deprivation of calcium infling. Inflammatory mediators derived by mast cells, especially TNF-α, IL-6, LTC₄, and PGD₂, play vital biological roles in allergic diseases. We found that Pts treatment reduced the release of these inflammatory mediators.

It is well known that LKB1/AMPK plays a crucial role in metabolic regulation and immunity. Recent studies have shown that AMPK is rapidly inhibited upon FcεRI activation, while AMPK activation can uncouple FcεRI-mediated Lyn signaling.^{40,48,49} To explore whether Pts can effectively alleviate mast cell activation by activating the LKB1/AMPK signaling pathway and inhibiting FcεRI activation, we detected key proteins involved in the LKB1-AMPK axis, MAPK family, IKK, and mTOR by western blot. As expected, during the formation of the FcεRI complex by FcεRI β and FcεRI γ, phosphorylation of LKB1, AMPK, and ACC was inhibited, whereas the phosphorylation of PLCγ1, p38, ERK, JNK, IKK, and mTOR was increased. In contrast, when LKB1/AMPK is activated by Pts before IgE/Ag stimulation, recruitment of FcεRI β and FcεRI γ subunits is blocked.¹⁷ At the same time, we also found that metformin, as an activator of LKB1-AMPK, also alleviated mast cell degranulation in allergic reaction by inhibiting FcεRI signaling. As known, metformin can not only regulate blood glucose and blood lipid but also have anti-inflammatory and anti-oxidant functions.^{50–52} Metformin is a drug, and it will cause side effects if used for a long time.⁵³ The difference is that Pts is not a drug but a natural plant ingredient. It can be used as a food or health product for a long time without any reports of toxic and side effects.

To further verify the relationship between Pts and LKB1/AMPK and FcεRI signaling, we used siRNA to knockdown LKB1. Akt is observed to be activated in response to IgE in mast cells.⁵⁴ It has been reported that Pts reduces acetaminophen-induced liver injury by activating the AMPK/Akt pathway.²⁶ Moreover, Liang et al. found that activation of AMPK/Akt/mTOR signaling promoted angiogenesis during the recovery of heat-denatured endothelial cells.⁵⁵ Jeong et al. found that activation of the AMPK/Akt/mTOR pathway triggering autophagy produced an anticancer effect.⁵⁶ However, the antiallergic effect of AMPK/Akt/mTOR on mast cells has not been reported. Interestingly, we found that FcεRI activation in RBL-2H3 cells could immediately increase phosphorylation of Akt, but Pts cannot suppress this increase. We speculate that Pts can temporarily increase p-Akt through the LKB1/AMPK pathway, which is also confirmed after the siRNA knockdown of LKB1. However, based on our data, we cannot rule out the involvement of other mechanisms in the degranulation effect of Pts. For example, Pts is known to scavenge reactive oxygen species, which could be another

mechanism underlying Pts degranulation inhibition other than LKB1. Future studies are warranted to further investigate other mechanisms of Pts.

According to the in vitro and in vivo experimental results, a schematic summary for the mechanisms of inhibitory effect of Pts on the FcεRI signaling pathway was drawn. As shown in Figure 7, Pts could suppress IgE-induced mast cell degranulation by activating LKB1/AMPK, which plays a vital role in regulating the pro-inflammatory cytokine secretion in allergic diseases.

In conclusion, we demonstrate that Pts may alleviate mast cell degranulation by activating the LKB1/AMPK signaling pathway. In the future, Pts may be expected to become a more effective product than metformin.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.9b07126>.

Effects of Pts on anti-DNP IgE-mediated PCA (PDF)

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Funding

This study was supported by the National Natural Science Foundation of China (nos. 81860729, 81560810, and 81660003 supported G.Y.; no. 81860549 supported L.Z.).

Notes

The authors declare no competing financial interest.

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