

Food Allergen–Induced Mast Cell Degranulation is Dependent on PI3K-Mediated Reactive Oxygen Species Production and Upregulation of Store-Operated Calcium Channel Subunits

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Received 24 November 2012; Accepted in revised form 8 April 2013

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Abstract

The importance of Ca^{2+} influx via store-operated calcium channels (SOCs) leading to mast cell degranulation is well known in allergic disease. However, the underlying mechanisms are not fully understood. With food-allergic rat model, the morphology of degranulated mast cell was analysed by toluidine blue stain and electron microscope. Ca^{2+} influx via SOCs was checked by Ca^{2+} imaging confocal microscope. Furthermore, the mRNA and protein expression of SOCs subunits were investigated using qPCR and Western blot. We found that ovalbumin (OVA) challenge significantly increased the levels of Th2 cytokines and OVA-specific IgE in allergic animals. Parallel to mast cell activation, the levels of histamine in serum and supernatant of rat peritoneal lavage solution were remarkably increased after OVA treatment. Moreover, the Ca^{2+} entry through SOCs evoked by thapsigargin was increased in OVA-challenged group. The mRNA and protein expressions of SOC subunits, stromal interaction molecule 1 (STIM1) and Orail (calcium-release-activated calcium channel protein 1), were dramatically elevated under food-allergic condition. Administration of Ebselen, a scavenger of reactive oxygen species (ROS), significantly attenuated OVA sensitization-induced intracellular Ca^{2+} rise and upregulation of SOCs subunit expressions. Intriguingly, pretreatment with PI3K-specific inhibitor (Wortmannin) partially abolished the production of ROS and subsequent elevation of SOCs activity and their subunit expressions. Taken together, these results imply that enhancement of SOC-mediated Ca^{2+} influx induces mast cell activation, contributing to the pathogenesis of OVA-stimulated food allergy. PI3K-dependent ROS generation involves in modulating the activity of SOCs by increasing the expressions of their subunit.

Introduction

During the last two decades, a dramatic increase in the occurrence of food allergy has been reported in worldwide [1–3]. The prevalence of food allergy to milk, eggs and peanuts is reported to be around 6–8% of children under the age of three [4, 5], while it is less common in adult population with a percentage of about 4% [6]. It has been documented that food allergy is primarily mediated by type I or Immunoglobulin E (IgE)-induced allergic reaction, although non-IgE-mediated allergy are gaining growing attention recently [7].

The role of mast cell in the pathogenesis of food allergy is well established. Mast cells are widely distributed in all layers of the mucosa (2–3% of the lamina propria cells in the gut are mast cells) and in the submucosal area [8]. Mucosal mast cells respond to both IgE-dependent (antigen) and non-IgE-dependent (bacterial toxins, neurotransmitters, etc.) stimulation and release a wide variety of bioactive mediators into adjacent tissues and exert their function in the allergic inflammation and in modulation of the gut function [9]. Besides an increased vascular permeability, mucosal oedema and contraction of smooth muscles, a diminished barrier integrity was observed

leading to an antigen-induced enhanced epithelial permeability [10]. These activated mast cells produce Th2-type cytokines, such as IL-3, IL-5 and IL-13 leading to the accumulation of eosinophils and other inflammatory cells relevant to allergic diseases [11].

The importance of calcium influx in mast cell activation and degranulation has been well recognized [12]. The degranulation of mast cell is Ca^{2+} dependent, and an increase in intracellular Ca^{2+} characterized by Ca^{2+} entry through store-operated calcium channels (SOCs) is essential for granule release [13–15]. Multiple mechanisms are involved in regulation of SOCs activity. It has recently been discovered that the two subunits, STIM1 and Orai1, play a vital role in both the signalling and the permeation mechanisms for Ca^{2+} influx through SOCs. Overexpression of STIM1 together with Orai1 caused a dramatic increase in store-operated Ca^{2+} entry in RBL cells [16]. Furthermore, SOC activation has been suggested to be linked to PI-3K signalling pathways, as well as reactive oxygen species (ROS) production, despite controversial. However, whether food allergen-induced mast cell activation is related to the regulation of intracellular Ca^{2+} signalling, and the underlying mechanism remain unknown.

In this study, using Brown-Norway rat food-allergic model, we aimed to investigate the involvement of Ca^{2+} signalling in food allergen-induced mast cell activation and degranulation and the underlying mechanisms. We found that Ca^{2+} entry through SOCs was increased in mast cells in the food-allergic animal model. SOC activation was related to PI3K-ROS-induced upregulation of STIM1 and Orai1 expression.

Materials and methods

Animals. Four-week-old female Brown-Norway rats were purchased from Vital River Laboratories (Beijing, China) and housed in groups of four per cage in a controlled environment with a photoperiod of 12-h light/12-h dark and a temperature of $20 \pm 2^\circ\text{C}$. Sanitary controls were performed for all major rodent pathogens, and the results of these tests were uniformly negative. All the animal experimental procedures were approved by the Animal Care and Use Committee of Shenzhen University and carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

Forty Brown-Norway rats were randomly divided into two groups: control group and ovalbumin (OVA, Sigma, USA) group. The control group received 1 ml Phosphate-buffered saline (PBS) per day for 6 weeks by gavage, while the OVA group was given 1 ml OVA (1 mg/ml) for same period as previously reported [17].

Rat peritoneal lavage solution preparation and mast cells staining. Rats were anaesthetized by inhalation of ether in

air and killed by decapitation. Skin around the lower abdomen was removed, and a small incision was made on the abdominal muscle to allow insertion of a trocar. Ten ml of lavage solution (D-Hanks) was administered through the trocar into the rat peritoneum. The rat peritoneum was massaged for 2 min, and the lavage solution was retrieved by a transfer pipette into a 15-ml conical tube. After centrifugation at 450 g for 5 min, the cell pellet was resuspended in 5 ml PBS for staining, while the supernatant was collected and stored at -80°C for measurement of cytokines and histamine. Typical mast cells in rat small intestine tissue or peritoneal lavage solution (RPLS) were stained with toluidine blue stain as previously described [18]. Briefly, 200 μl peritoneal lavage fluids was dried in the air on cromolyn sodium-pretreated slides and then covered with several drops of staining solution (toluidine blue stain dissolved in 70% ethanol). After 90 s, the staining solutions were washed away quickly with the running tap water, and the stained cells were examined and counted under light microscope (Olympus, Japan).

Rat peritoneal mast cell isolation and *in vitro* allergic model. The BN rats were sacrificed after anaesthetized by inhalation of ether in air. Rat peritoneal mast cells (RPMCs) were obtained by peritoneal lavage and purified by density gradient fractionation as described previously [19, 20]. Isolated RPMCs preparations contained >98% mast cells and at least 98% of these cells were viable as checked by metachromatic staining in 0.05% toluidine blue. The cells were then used for the following RT-PCR, Western blot, Ca^{2+} image and immunofluorescence experiments of SOCs subunits.

For a mechanistic study, *in vitro* allergic model was re-established as follows: isolated RPMCs from control group were cultured at density of 2×10^6 cells per well for 30 min in 24-well tissue culture plates with DMEM (GIBCO) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin. Then, the cells were divided into control, OVA, Wortmannin (Sigma, USA) and Ebselen (Sigma, USA) group. The cells in Wortmannin group were pretreated with 100 nM Wortmannin for 15 min, while Ebselen group were pretreated with 100 μM Ebselen for 30 min. After that, all the groups of cell, except control group, were sensitized by 30% RPLS (diluted by DMEM) from OVA-treated rats for 6 h. All the cells were then challenged by 10 $\mu\text{g}/\text{ml}$ OVA for 1 h and used for the following experiments of RT-PCR, Western blot and Ca^{2+} imaging of SOCs subunits.

Ca^{2+} imaging by confocal microscope. Intracellular Ca^{2+} signal was measured as described previously with minor modification [21]. Rat peritoneal mast cells (RPMCs) were incubated with 5 μM Ca^{2+} fluorescent probe fluo-4 AM (Invitrogen, CA, USA) for 30 min at room temperature. After washing with Tyrode's buffer for three times, the dye inside the cells were allowed to de-esterify for 30 min at

37 °C. It has been determined that nearly 95% of the fluorescent dye was retained in the cytoplasm. Fluorescent images of Ca^{2+} were obtained using Olympus 1000 confocal microscope with 40 × oil immersion lens (NA 1.3; Olympus, Japan). Fluo-4 signal was excited at 488-nm and emitted at >505 nm. Frame-scan images were acquired at sampling rate of 15 ms per frame and 20 s per interval.

Image data were analysed offline using FV10-ASW 2.1 software (Olympus, Japan). A selected image from each image set was used as a template for designating the region of interest (ROI) within each cell. The integrated intracellular Ca^{2+} concentration was determined by calculating $\Delta F/F_0$. F_0 was defined as the mean basal fluorescence intensity of the dye recorded during the first 5–10 scanning frames, when the cells were under rest conditions. ΔF denotes ($F - F_0$), where F is the temporal fluorescence intensity. The $\Delta F/F_0$ values within each ROI were plotted as a function of time (see Fig. 5 for typical time-courses of Ca^{2+} response to thapsigargin or DNP-BSA stimulation in single RBL-2H3 cell). The amplitude of the Ca^{2+} response within each cell was quantified as the highest $\Delta F/F_0$ level reached during the measurement period, which was averaged over all cells within each group.

Quantitative real-time RT-PCR. Total RNAs were extracted from RPMCs using TRIzol Reagent (Invitrogen, CA, USA) according to manufacturer's instructions. Reverse Transcription was conducted in a 20 μl reaction mixture (ReverTra-Plus-RT-PCR Kit, Toyobo), and cDNA was synthesized from 2 μg of total RNA. The prepared cDNA was further analysed for gene expression by real-time RT-PCR with gene-specific primers. The primer sequences for different genes were as follows: Orail: forward, 5'-ACGTCCACAACCTCAACTCC-3'; reverse, 5'-GGTATTCTGCCTGGCTGTCA-3'. STIM1: forward, 5'-GGCCAGAGTCTCAGCCATAG-3'; reverse, 5'-TAGTCGCACCTCCTGGATAC-3'. TLR4: forward, 5'-TGC TCAGACATGGCAGTTTC-3'; reverse, 5'-TCAAGGCTT TTCCATCCAAC-3'. GAPDH: forward, 5'-TCACCATC TTCCAGGAGCGA-3'; reverse, 5'-TGCTGGTGAAGCC GTAACAC-3'. Real-time PCR was performed using Bio-Rad SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following cycling conditions: 5 min at 94 °C, followed by 45 cycles of 94 °C for 30 s, 54.3 °C for 30 s and 72 °C for 45 s. RNA abundance was expressed as $\Delta\Delta\text{Ct}$, and the fluorescence signals of target gene expression were normalized to that of the internal control (GAPDH).

Western blot. Total cell lysates were extracted from RPMCs by Laemmli buffer. Equal amount of proteins were loaded, separated on 10% SDS-PAGE before being transferred to polyvinylidene difluoride (PVDF) membranes, and then probed with primary antibody: anti-Orail (1:1000, Abcam, UK), anti-STIM1 (1:1000, Abcam, UK) and anti-GAPDH (1:1500, Abcam, UK). The membranes were washed for three times and incubated with corresponding

secondary antibodies at room temperature for 1 h. The signals of immune complexes were visualized using enhanced chemiluminescence (ECL) system. Quantitative analysis was performed after densitometric scanning, and the results were normalized to internal control GAPDH.

Fluorescence imaging of STIM1 translocation: Immunofluorescence staining of STIM1 translocation in RPMCs was performed as described previously [22]. Briefly, after fixation, permeabilization and blocking, the cells were incubated with rabbit anti-rat STIM1 antibody (1:100 dilution) at 4 °C overnight. Subsequently after three washes with PBS, the cells were incubated with FITC-conjugated secondary antibody (goat anti-rabbit IgG, 1:1000) for 1 h at room temperature. Signals were then detected by Olympus 1000 confocal microscope (Olympus, Japan). Control staining was carried out with non-immune IgG used at the same concentration as the primary antibody. Six randomly selected fields in each sample in an individual experiment were scored, and at least three independent experiments were performed.

Enzyme-linked immunosorbent assay (ELISA). The contents of tumour necrosis factor- α (TNF α), interleukin-4 (IL-4), interleukin-10 (IL-10), interferon- γ (IFN γ) and histamine in rat peritoneal lavage solution (RPLS) and serum were assayed by commercial ELISA kits using paired antibodies according to the manufacturer's instructions. The kits for detecting TNF- α , IL-4, IL-10 and IFN- γ were bought from eBioscience (USA), and the kit for detecting histamine was bought from R&D Inc. (Minneapolis, MN, Minneapolis, MN, USA). Serum IgE levels were also detected using a commercial ELISA kit (BD Biosciences Pharmingen, San Jose, CA, USA), following the manufacturer's instructions.

Statistics. Data are presented as means $\pm$ SD. When two comparisons were obtained, Student's unpaired two-tailed t test was used. When multiple comparisons were obtained, the analyses consisted of one-way ANOVA for repeated measures and Student–Newman–Keuls multiple comparison test. A value of $P < 0.05$ was considered to be statistically significant.

Results

OVA-induced food allergy increased Th2 cytokines release and IgE production

In the present study, we used OVA oral sensitization to establish food-allergic model in Brown-Norway rats as previously reported [17]. The cytokine levels in RPLS were measured by ELISA. The results showed that type Th2 cytokines (IL-4, 11.8 ± 1.52 pg/ml; IL-10, 101.3 ± 15.37 pg/ml) were significantly higher than those in control groups (IL-4, 3.73 ± 0.18 pg/ml; IL-10, 61.66 ± 8.33 pg/ml; Fig. 1A). However, the concentrations of type Th1 cytokines, including IL-2 and IFN γ , were similar to those in control group. The above

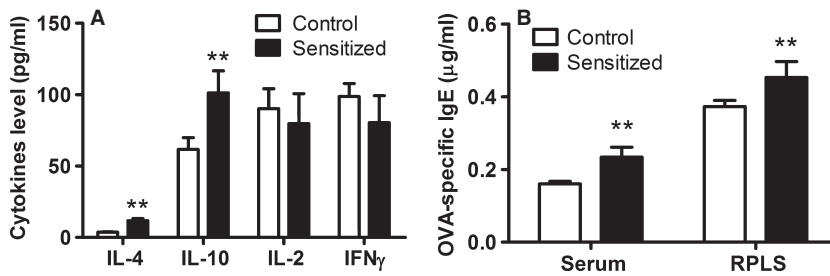


Figure 1 Evaluation of ovalbumin (OVA)-induced food-allergic model. (A) The cytokine levels in rat peritoneal lavage solution (RPLS) were analysed by ELISA, $n = 8$. (B) Statistical analysis of OVA-specific IgE in serum (left panel) and RPLS (right panel), which were collected from rats administered with or without OVA. **, $P < 0.01$ versus control.

results indicate that the ratio of Th1/Th2 was decreased, and the balance of Th1/Th2 was skewed in OVA-induced food-allergic model. ELISA analysis showed that the concentrations of OVA-specific IgE in both serum and RPLS were significantly higher (0.23 ± 0.03 versus ctrl 0.16 ± 0.01 $\mu\text{g/ml}$ in serum; 0.45 ± 0.04 versus ctrl 0.37 ± 0.01 $\mu\text{g/ml}$ in RPLS) in OVA-induced food-allergic group (Fig. 1B).

Food allergen-induced mast cell activation in small intestine

The number and morphology of the mast cells in rat small intestine tissues (data not shown) or RPLS were examined by toluidine blue stain. In sensitized group, the mast cells were much bigger, with more shrink on the cell membrane, bubbles in the cytoplasm and degranulation vehicles around the cells (Fig. 2A). Furthermore, ultra-structure analysis of mast cells by transmission electron microscope showed that the cell membrane was obscure, and degranulation vehicles was less evenly distributed in the cytoplasm of mast cells (Fig. 2A). The number of mast cells was significantly increased in OVA-treated RPLS (Fig. 2B). The ratio of mast cell degranulation as indicated by vehicles (at least five) around the cells was also dramatically increased by ~ 3 fold (Fig. 2B). Mast cell degranulation was further confirmed by increased histamine levels in serum and RPLS (Fig. 2C).

Ca²⁺ entry through SOCs was increased in mast cells in food allergy

It has been suggested that an increase in intracellular Ca²⁺ through SOC channel is essential for mast cell degranulation [13]. We therefore examined whether food allergen-induced mast cell activation is related to stimulation of Ca²⁺ mobilization. As shown in Fig. 3, the TG-evoked Ca²⁺ influx was dramatically enhanced in OVA-sensitized rat peritoneal mast cells, suggesting mast cell activation in the food-allergic model is related to upregulation of Ca²⁺ entry through SOCs. STIM1 and Orai1 are the two subunits of SOCs [23, 24]. Overexpression of STIM1 and Orai1 caused a significant increase in store-operated Ca²⁺ entry in RBL cells [16]. We thus examined the expression levels of both subunits. The results show that the mRNA (Fig. 3A,B) and protein levels (Fig. 3C,D)

of both subunits were significantly increased in allergic animals as compared with controls (all $P < 0.01$). Furthermore, immunofluorescence study revealed that the STIM1 subunits were translocated to the cell membrane, which is required for the activation of SOCs in OVA group, while it was evenly distributed in cytoplasm in control group (Fig. 4). Collectively, these data indicate that OVA-induced food allergy increased SOCs activity by enhancing transcription and expression of SOCs subunits, as well as increasing SOCs activity.

PI3K-mediated ROS production regulated SOCs activity by increasing STIM1 and Orai1 expression

Reactive oxygen species production in RPMCs isolated from control or allergic animals was examined by ELISA. The results demonstrated that ROS production in allergic mast cells was increased by 1.5-folds as compared with controls (Fig. 5A). Administration with ROS scavenger Ebselen (100 μM , 30 min) to OVA-challenged RPMCs reduced ROS production by $\sim 30\%$ (Fig. 5A). In parallel, clearance of intracellular ROS by Ebselen decreased histamine release by $\sim 30\%$ (Fig. 5B). Similarly, OVA challenge-induced Ca²⁺ increase through SOCs in activated mast cell was decreased by 30% by Ebselen treatment (Fig. 5C,D). The results indicate that mast cell activation is partially attributed to increased ROS production. Quantification of the protein levels of Orai1 and STIM1 demonstrated that Ebselen reduced both protein expressions by $\sim 40\%$ and $\sim 30\%$, respectively (Fig. 5E,F), suggesting that increased ROS acts through upregulating the expression of SOC subunits to increase SOC activity.

Previous study has shown that cross-linking of Fc ϵ R1 activates PI3K signalling pathway, leading to intracellular ROS production [25]. To explore whether OVA challenge-induced ROS production and subsequent activation of SOCs are related to PI3K activation, we explored the effect of PI3K inhibitor Wortmannin on ROS production and Ca²⁺ signalling in OVA-activated mast cells. The results demonstrated that Wortmannin (100 nM, 15 min) pretreatment significantly decreased intracellular ROS production by $\sim 30\%$. Mast cell activation-induced histamine release was similarly reduced ($\sim 30\%$) by inhibiting PI3K pathway. With the reduction of ROS, Ca²⁺ increase through SOCs in OVA-activated mast cells was diminished

Figure 2 Mast cells were activated in ovalbumin (OVA)-challenged group. (A) The mast cells in rat peritoneal lavage solution (RPLS; upper panel, magnification 400 $\times$) were identified by toluidine blue stain, and the ultrastructure of mast cells in intestine tissue was further analysed by transmission electronic microscope (lower panel, magnification 3200 $\times$). Mast cells were considered degranulated if at least five granules appeared outside the cell body. Arrows indicate mast cell. (B) Summary cell numbers (upper panel) and ratio of degranulation (lower panel) of mast cells. (C) The release of histamine in serum (left panel) or RPLS (right panel) was measured by ELISA. *, $P < 0.05$; **, $P < 0.01$ versus control.

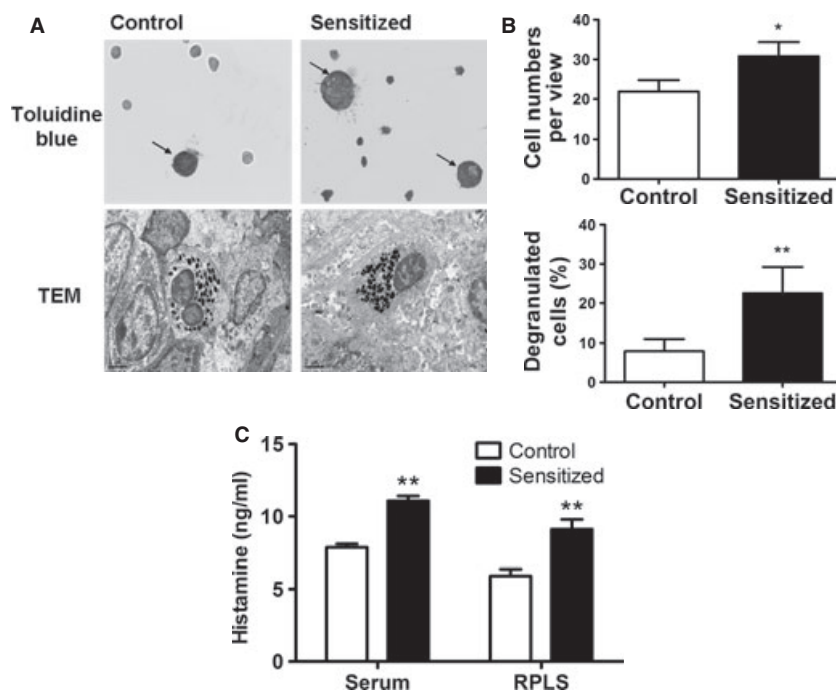
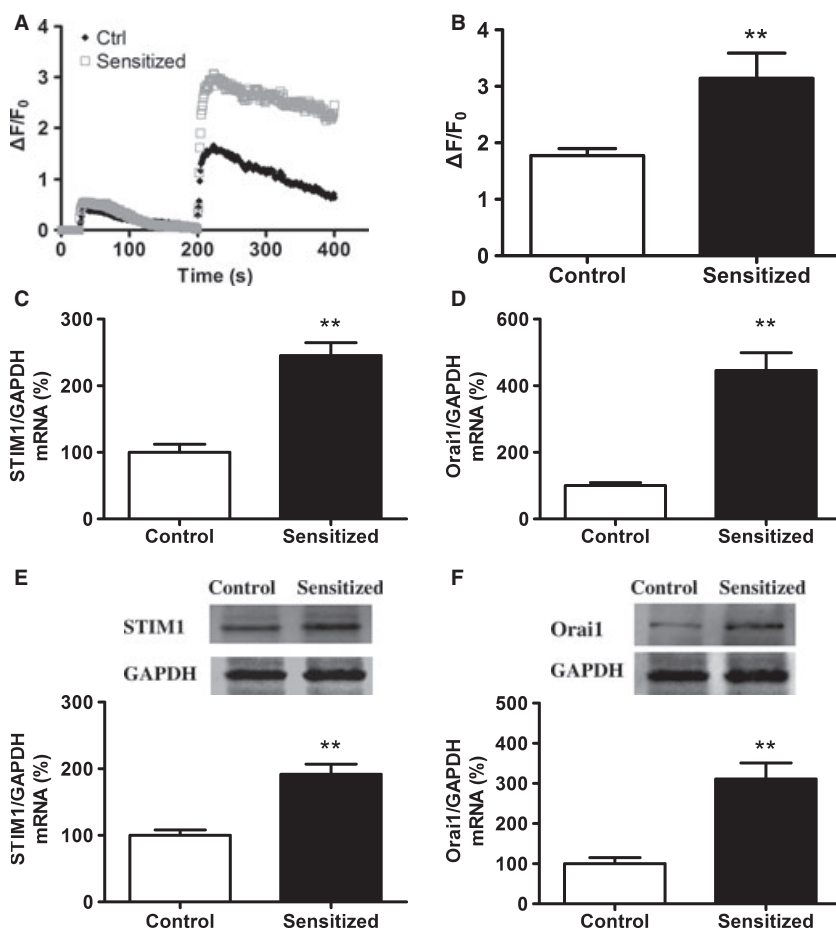


Figure 3 Ovalbumin (OVA)-induced food allergy upregulated Ca^{2+} entry through store-operated calcium channels (SOCs). Rat peritoneal mast cells (RPMCs) were isolated from BN rat treated with or without OVA. Intracellular Ca^{2+} was indicated by fluo-4 fluorescence. Total cell numbers are 40–50 for each group, and the cells were from four independent experiments. (A) Typical responses of TG-evoked Ca^{2+} entry through SOCs in RPMCs. (B) Averaged peak amplitude of Ca^{2+} increase as recorded in A. C and D, OVA-induced food allergy significantly increased mRNA levels of STIM1 (C) and Orai1 (D). E and F, representative images and quantification of protein levels of STIM1 (E) and Orai1 (F). $N = 8$. **, $P < 0.01$ versus control.



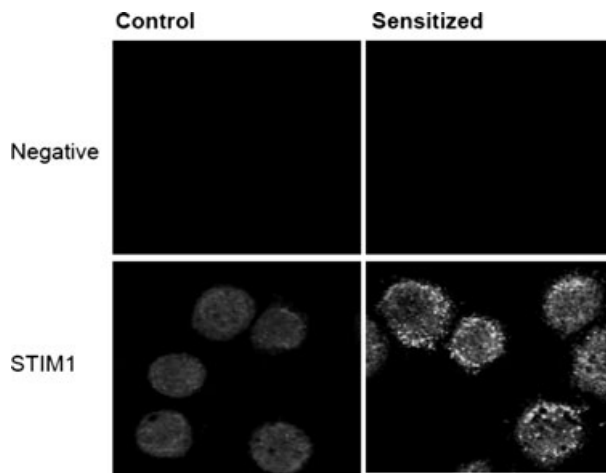


Figure 4 Translocation of STIM1 to the membrane of rat peritoneal mast cells (RPMCs). The localization of STIM1 protein in rat peritoneal mast cell was checked by immunofluorescence. Representative images from three independent experiments (at least 100 cells) are shown. Magnification, 1000 $\times$.

by $\sim 30\%$ (Fig. 6A,B). Consistently, the protein expressions of Orai1 and STIM1 were attenuated by $\sim 40\%$ and $\sim 30\%$, respectively (Fig. 6C,D). We also found that inhibition of PI3K pathway reduced mast cell activation-induced histamine release ($\sim 30\%$) and intracellular ROS production ($\sim 30\%$). The results indicate that PI3K-mediated ROS generation is involved in the regulation of SOCs activity and mast cell activation under food-allergic condition (Fig. 6E,F).

Discussion

Previous studies have demonstrated that mast cells play a critical role in allergic diseases. Using OVA-stimulated food-allergic rat model, we revealed that mast cells were recruited and activated in the damaged intestinal tissues and peritoneal lavage, and Th2 cytokines and IgE were significantly increased, confirming the notion that mast cells contribute to the pathogenesis of food allergy. In this study, we demonstrated that the underlying mechanism for mast cell activation in the food-allergic mouse model is related to increased Ca^{2+} entry through SOCs. Furthermore, we found

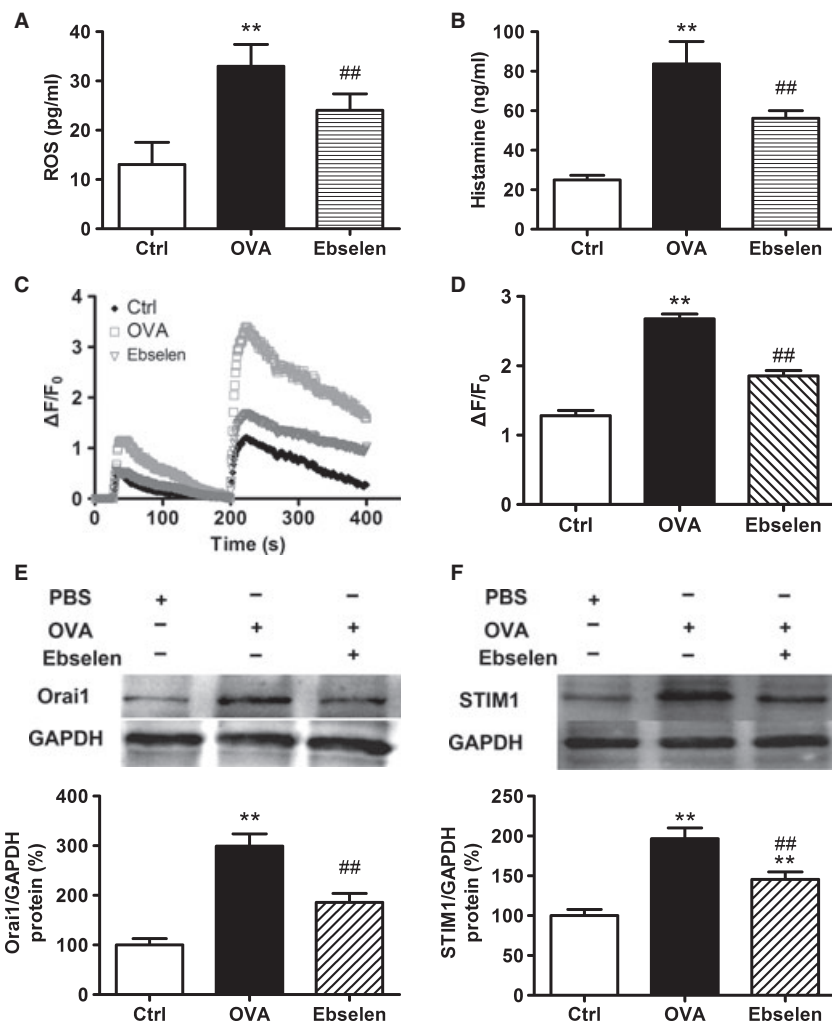


Figure 5 Reactive oxygen species (ROS) production contributed to intracellular Ca^{2+} rise through store-operated calcium channels (SOCs). (A) Intracellular ROS concentration in control and ovalbumin (OVA)-activated rat peritoneal mast cells (RPMCs) treated with (Ebselen) or without Ebselen (OVA). (B) Ebselen dramatically attenuated OVA-induced histamine release from RPMCs. (C) typical responses of TG-evoked Ca^{2+} entry through SOCs in RPMCs pretreated with or without Ebselen. (D) averaged peak amplitude of Ca^{2+} increase as recorded in C. E and F, representative images and quantification of protein levels of STIM1 (E) and Orai1 (F) in RPMCs pretreated with or without Ebselen. $N = 8$. **, $P < 0.01$ versus ctrl group; ##, $P < 0.01$ versus OVA group.

that OVA stimulation increased intracellular ROS production in mast cells through activation of phosphoinositide 3-kinase (PI3K) pathway, which results in upregulation of the expression levels of the major subunits of SOC, Orai1 and STIM1, leading to the enhancement of SOC activity and subsequent mast cell activation.

Food allergy is one type of adverse reactions to non-toxic food that involves an abnormal immunological response to specific protein(s) in food. Allergens from egg seem to be one of the most frequent causes of food-allergic reaction as reported [26]. In the present study, we use OVA, which comprise 50% of the protein in egg white, to induce food allergy as previously reported [17, 27, 28]. According to our results, the food-allergic model in Brown-Norway rats has been successfully re-established. The OVA-challenged rat showed typical allergic appearances, including puffiness and redness around the eyes and mouth, diarrhoea, pilar erecti, reduced activity and/or decreased activity with increased respiratory rate and cyanosis around the mouth and tail.

Because Putney firstly proposed the hypothesis that intracellular Ca^{2+} concentration rise in activated mast cell is through SOCs [29], it has been well established that an increase of intracellular Ca^{2+} characterized by Ca^{2+} entry through SOCs is essential to mast cell degranulation [13–15]. Mast cells play a key role in allergic and inflammatory reactions. Mast cells and some tumour cell lines such as RBL-2H3 express the high-affinity IgE receptor (Fc ϵ RI) on their cell surface. Fc ϵ RI is a member of the multichain immune recognition receptors (MIRRs), including T- and B-cell receptor. With regard to OVA-challenged and IgE-mediated mast cell degranulation, Fc ϵ RI aggregation activates phospholipase C γ to increase IP $_3$ generation. The IP $_3$ causes Ca^{2+} release from the endoplasmic reticulum through IP $_3$ receptors, which consequently results in a large amount of Ca^{2+} influx via SOCs, leading to mast cell degranulation. In the present study, we demonstrated for the first time that parallel to enhancement of food allergen-induced mast cell degranulation, OVA-mediated Ca^{2+} entry through SOCs was increased. Given that increasing Ca^{2+} entry through SOCs enhances mast cell degranulation [20], we conclude that increase in Ca^{2+} entry through SOCs contributes to food allergen-mediated mast cell degranulation.

The two membrane proteins, STIM1 and Orai1, have been shown to be essential for the activation of SOCs [16]. Overexpression of Orai1 together with STIM1 has been suggested to upregulate Ca^{2+} entry through SOCs upon stimulation. In this study, we found that both mRNA and protein expressions levels of Orai1 and STIM1 in mast cells were increased in OVA-sensitized animals, which is proposed to be an important reason accounting for the increase in SOC-mediated Ca^{2+} entry and mast cell activation. It has been suggested that the N-terminal of STIM1 is glycosylated and translocated from endoplasmic reticulum to the cell

membrane when the calcium store is depleted, which process is required for activation of SOCs [30]. This is in line with our study as the translocation of STIM1 protein to activated mast cell membrane in OVA-sensitized mast cells. Therefore, our study demonstrates for the first time that overexpression and activation of SOCs contributes to enhancement of Ca^{2+} entry through SOCs in food-allergic rats.

Activated mast cell can release a diverse array of biologically active products, including preformed granule contents, the *de novo* synthesis of eicosanoids, cytokines, chemokines and free radicals (such as ROS) [31]. Large amount of ROS has been demonstrated to generate in inflammatory cells during asthma, but little information is known in the situation of food allergy. A number of studies report that ROS are involved in the signals leading to degranulation and cytokine secretion in mast cells [32, 33]. In this study, we found that ROS production was significantly increased in the peritoneal lavage solution. Using Ebselen to partially scavenge ROS production (mainly hydrogen peroxide), Ca^{2+} entry through SOCs was inhibited. Concomitantly, the protein expressions of Orai1 and STIM1 were similarly decreased. These data collectively indicate that ROS generation is involved in the regulation of SOCs activity.

Reactive oxygen species induction is often accompanied by the activation of PI3K, a lipid kinase that can support cell growth, migration and survival [34–36]. Inhibition of PI3K with pharmacological or genetic methods indeed abolished ROS generation induced by chemokine/cytokine/growth factors [37–41]. The regulation of PI3K-mediated ROS production on Ca^{2+} signalling has been reported in cultured mast cell model, involving ERK-dependent or independent pathways [25, 42]. In the present study, PI3K-specific inhibitor Wortmannin decreased intracellular ROS generation in mast cell under food-allergic condition. Accordingly, Ca^{2+} entry through SOCs and the expression levels of both subunits of SOCs were significantly suppressed by inhibition of PI3K. Therefore, activation of PI3K pathway is an important mechanism, inducing intracellular ROS production in food-allergic rats. Of note, Wortmannin only partially inhibited ROS production, suggesting other mechanism(s) (such as activation of 5-lipoxygenase and cyclooxygenase-1 [43]) participate in food allergen-induced ROS generation. Further studies are warranted to address the above problems. A schematic diagram for the involvement of PI3K-ROS pathway in enhancement of SOC activity and subsequent mast cell activation upon food allergen stimulation was proposed in Fig. 7.

In summary, in OVA challenge-induced food-allergic rats, we demonstrated for the first time that PI3K-mediated ROS production causes enhancement of Ca^{2+} entry through SOCs by upregulating SOC subunits and activity, thereby leading to subsequent mast cell activation and degranulation. Inhibiting PI3K-ROS pathway has a

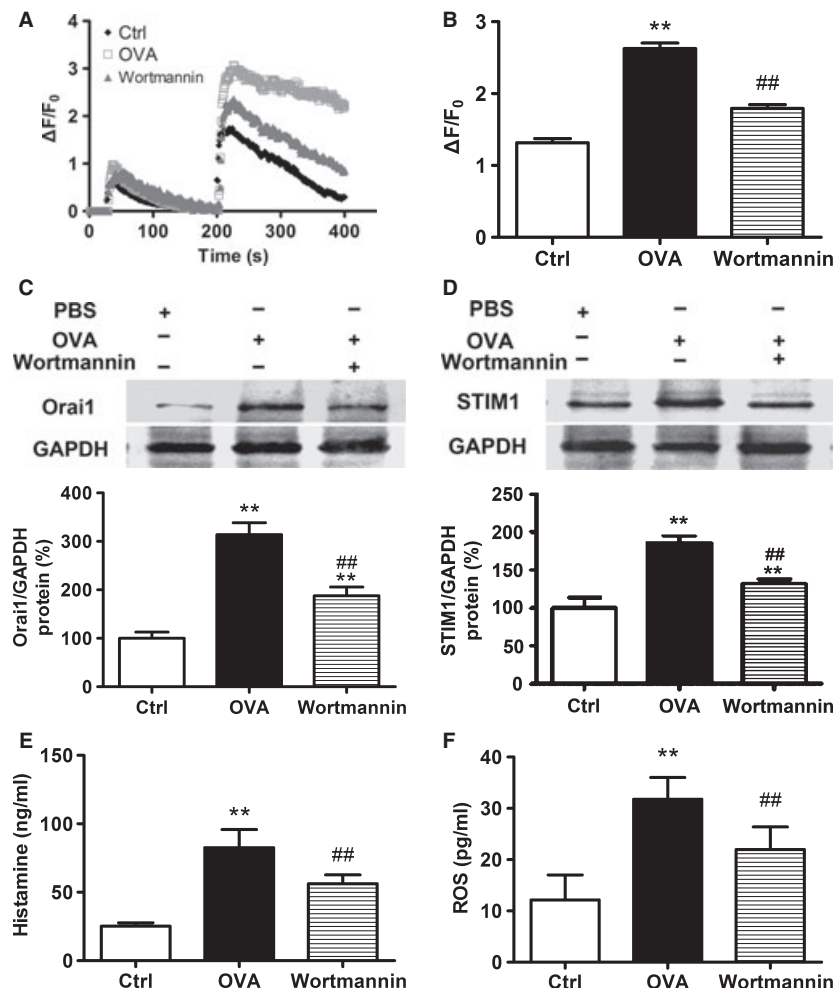


Figure 6 PI3K-mediated reactive oxygen species (ROS) production contributed to increased Ca^{2+} influx through store-operated calcium channels (SOCs). (A) typical responses of TG-evoked Ca^{2+} entry through SOCs in control and ovalbumin (OVA)-activated rat peritoneal mast cells (RPMCs) pretreated with (Wortmannin) or without Wortmannin (OVA). (B) averaged peak amplitude of Ca^{2+} increase as recorded in A. C and D, representative images and quantification of protein levels of STIM1 (C) and Orai1 (D) in RPMCs pretreated with or without Wortmannin. (E) PI3K-specific inhibitor, Wortmannin dramatically attenuated OVA-induced histamine release from RPMCs. (F) Intracellular ROS concentration in RPMCs treated with or without Wortmannin. $N = 8$. **, $P < 0.01$ versus ctrl group; ##, $P < 0.01$ versus OVA group.

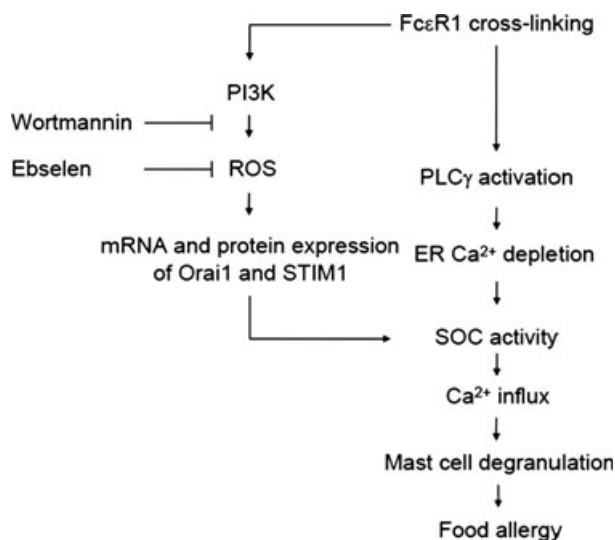


Figure 7 Schematic diagram. PI3K-mediated reactive oxygen species (ROS) production increases Orai1 and STIM1 transcripts and protein expression levels, leading to enhancement of Ca^{2+} influx through store-operated calcium channels (SOCs) and mast cell degranulation in food-allergic model. ER, endoplasmic reticulum; PLCγ, phospholipase Cγ.

potential therapeutic effect on the treatment of food allergy.

Acknowledgment

This work was supported by grants from the Natural Science Foundation of China (No. 81271950 to Q.J., 31101280 to H.H.), Key Laboratory Construction Program of Shenzhen (No. SW201110010), Basic Research Foundation of SZ (No. JC201005250059A, JCYJ201206131 15535998) and Basic Research Program of Shenzhen University (No. 201101 to Z.L.).

Conflict of interest

The authors have no conflict of interest to declare.

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