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ORIGINAL ARTICLE

Denatonium benzoate promotes IgE-mediated mast cell degranulation and ovalbumin-induced allergic diarrhea in BALB/c mice

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Abstract

Background: Denatonium benzoate (DB), one of the most bitter compounds known to man, is used for alcohol denaturation. Some reports have demonstrated that asthmatic symptoms are associated with DB exposure, but the possible links between DB and immunoglobulin E (IgE)-mediated allergy susceptibility have not yet been examined.

Objective: This study investigated the effects of DB on *in vitro* IgE-mediated mast cell degranulation and food allergy in BALB/c mice.

Methods: IgE-sensitized rat RBL-2H3 cells (a basophilic leukemia mast cell line) and human KU812 cells (a basophilic cell line) and mice with ovalbumin (OVA)-induced allergy were treated with DB. Histamine release, calcium ion (Ca^{2+}) influx, phosphorylated spleen tyrosine kinase (p-Lyn) and phosphorylated phospholipase C-γ (p-PLCγ) levels, OVA-specific IgE, anaphylactic symptoms, and the cell-surface expression of the high-affinity IgE receptor α-subunit (FcεRIα) on mast cells were evaluated.

Results: DB increased histamine release, Ca^{2+} mobilization, and p-Lyn and p-PLCγ levels in IgE-mediated activated RBL-2H3 and KU812 cells, and enhanced the cell-surface expression of FcεRIα messenger RNA (mRNA). In mice, DB increased the severity of OVA-induced anaphylactic and diarrheic symptoms, the mucus thickness in the jejunum, histamine and OVA-specific IgE levels, and FcεRIα mRNA in isolated mucosal mast cells.

Conclusion: Our work indicates that DB promotes IgE-mediated mast cell degranulation and OVA-induced allergic diarrhea in BALB/c mice, providing evidence that exposure to DB promotes allergy susceptibility.

Key words: Denatonium benzoate, IgE, FcεRIα, mast cell degranulation, allergic diarrhea

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Introduction

Mast cells are key effector cells in immunoglobulin E (IgE)-mediated allergy. They express FcεRI, the high-affinity IgE receptor that binds IgE through the extracellular portion (FcεRIα). There are two distinct interaction sites: the Cε3 domain, in direct contact with the receptor, and the Cε4 domain, forming a heavy chain dimerization interface.[1] Antigen crosslinking of FcεRI initiates a broad range of events including immediate induction of signaling cascades leading to degranulation, whereby granule-stored mediators such as histamine, serotonin, and proteases are released, and the production of arachidonic acid is increased. This results in the generation of the characteristic symptoms of an acute allergic reaction.[2]

The prevalence of allergic disease has increased dramatically in the last few decades. Multiple environmental factors, most notably chemicals, have been associated with the increased incidence of allergies.[3] Denatonium benzoate (DB), the most bitter compound known to man, is intentionally applied to a wide range of products (e.g., antifreeze, finger paints, and detergents) to prevent intended or accidental consumption.[4] This compound is also found in numerous cosmetics and other personal care products due to the utilization of denatured alcohol.[5] In agreement with its usage patterns, DB was recently identified as a wastewater-borne micropollutant in the environment, reaching concentrations up to 400 ng L^{-1} in surface water.[6] However, DB is generally considered very safe: Animal studies have shown that it is not carcinogenic, genotoxic, or mutagenic.[7] Although some reports have demonstrated an association between asthmatic symptoms and exposure to DB,[8–10] the linkage between DB and allergy susceptibility has not been formally validated.

Here, we hypothesized that DB has potent effects on IgE-mediated mast cell degranulation and allergy induction. To test this hypothesis, we investigated the influence of DB on IgE-mediated mast cell functions *in vitro* using RBL-2H3 and KU812 cells and *in vivo* using a BALB/c mouse model of ovalbumin (OVA)-induced allergic diarrhea. After DB treatment, we examined allergic mediator release, calcium ion (Ca^{2+}) influx, p-Lyn and p-PLCγ levels, anaphylactic symptoms, serum OVA-specific IgE levels, and FcεRIα messenger RNA (mRNA) expression on mast cells. Our findings provide support for IgE-mediated allergy susceptibility associated with exposure to DB.

Materials and methods

Chemicals and cell lines

RBL-2H3 and KU812 cells were purchased from the Type Cell Culture Collection of the Chinese Academy of Science (Wuhan, China). DB, OVA, a monoclonal anti-dinitrophenyl IgE antibody (anti-DNP IgE), and 4-nitrophenyl-N-acetyl-β-D-glucosaminide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dinitrophenyl–human serum albumin (DNP–HSA) was obtained from Biosearch Technologies, Inc. (Novato, CA, USA). The antibodies against Lyn and PLCγ were purchased from Boster (Wuhan, China). Antibodies for p-Lyn and p-PLCγ were obtained from Bioss (Beijing, China) and HuaAn (Hangzhou, China), respectively. Cell culture medium (RPMI 1640) was from Thermo Fisher Scientific (Waltham, MA, USA), and the cell counting kit-8 (CCK-8) was procured from ApeXBio (Houston, TX, USA). The vendors for the other reagents are included with the relevant assays.

Mice

Female BALB/c mice were supplied by the Department of Animal Science, Nanchang University (Nanchang, China; permission number SYXK [Gan] 2017-00021). In each animal experiment, mice (6–7 weeks old and weighing 20.0 ± 2.0 g) were randomly divided into several groups (n = 8 per group). All mice used in this study were cared for according to the approved guidelines of the Care and Use of Laboratory Animals guidelines (NIH Publication No. 86-23, Revised 1996), and all experimental procedures were approved by the Animal Care Review Committee, Nanchang University (approval number NCU2018-0227).

Human sera preparation and ethics statement

Human sera were prepared as described previously.[11] The sera were pooled from 10 patients with an egg allergy. Specific OVA-IgE and total IgE levels were determined (specific IgE level > 1 IU/mL, total IgE level > 100 IU/mL) with an ImmunoCAP 100E instrument (Phadia AB, Uppsala, Sweden). Sera from non-allergic individuals (n = 4) were also pooled and used as negative controls. Human sera were collected under the approval of the Nanchang University Ethic Committee.

Treatment of mast cell lines, cell viability, and cytotoxicity assays

RBL-2H3 and KU812 cells were grown at 37°C in RPMI-1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (Invitrogen, CA, USA). Cells were incubated with various concentrations of DB for 24 h. Meanwhile, RBL-2H3 cells were sensitized overnight with 500 ng/mL anti-DNP IgE and KU812 cells were sensitized with 1:5 dilutions of patient serum, before challenging the cells for 30 min with 50 μg/mL DNP–HAS (RBL-2H3 cells) or 50 μg/mL OVA (KU812 cells). The control group received no DB treatment, while the naïve group was only treated with vehicle substances, whereas cells were treated with DB only to evaluate whether DB alone has any specific effects on mast cell degranulation. Prior to experiments, a 1 M DB stock solution was prepared in phosphate-buffered saline; the stock was diluted in RPMI-1640 to obtain the desired DB concentration.

The CCK-8 assay was performed according to manufacturer's instructions. Briefly, cells were cultured in 96-well plates and then treated with varying concentrations of DB for 2 h. Then, cell supernatants were removed, followed by incubation with CCK-8 solution for 4 h before measuring absorbance intensities at 450 nm in a microplate reader (Thermo Fisher Scientific).

Measurements of histamine, intracellular Ca^{2+}, and OVA-specific IgE

Degranulation of mast cells was determined by measuring the release of the granule marker histamine in the cell culture supernatant or mouse sera with an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Mouse OVA-specific IgE from sera were also measured with an ELISA kit (BioLegend Inc., San Diego, CA, USA).

A fluorescence-based assay was used to analyze intracellular Ca^{2+}, as described previously.[12] After DB and antibody treatment, about 1×10^6 cells were pulsed with 5 μM Fluo-3/AM containing 0.05% PluronicF127 for 30 min before adding DNP–HAS or OVA. After 5 min, the fluorescence intensity was recorded at 488 nm (excitation wavelength) and 526 nm (emission wavelength) using a Varioskan Flash microplate reader (Thermo Fisher Scientific).

Immunofluorescence microscopy and image analysis

RBL-2H3 cells were cultured on coverslips while KU812 cells were cytocentrifuged onto silane-coated glass slides, followed by processing for immunofluorescence microscopy, with minor modifications of previous methods.[13] Cells were incubated with the FcεRIα antibody at 4°C overnight using the manufacturer's recommended dilution before detection with fluorophore-labelled (Alexa Fluor 488 and 594) secondary antibodies (Invitrogen). 4′,6-Diamidino-2-phenylindoledihydrochloride (DAPI; Invitrogen) was used to counterstain nuclei. Digital images were captured with an Eclipse Ci-L light microscope with an attached Digital Sight DS-Fi2 camera (Nikon, Tokyo, Japan), and processed and analyzed using Image-Pro Plus 6.0.

Animal protocol

Mice were tested for their susceptibility to OVA-induced allergy using a previously published protocol.[14] Briefly, mice were sensitized twice, 2 weeks apart, by intraperitoneal injection of 50 μg of OVA in the presence of 1 mg of aluminum potassium sulfate adjuvant ($AIK(SO_4)_2 \cdot 12H_2O$; A-7210, Sigma-Aldrich). Two weeks later, the mice were subjected to intragastric gavage with 250 μL of sterile saline that contained up to 50 mg of OVA every other day from day 28. To evaluate the effects of DB on OVA-induced allergy, the mice were treated intragastrically with 2.5 mM DB in 250 μL of sterile saline every other day from day 25 to day 42 (**Figure 1**). Mice treated with OVA only was used as the control group, whereas the naïve group was treated with just the vehicles. Additionally, some mice were treated with DB alone to evaluate whether DB alone has any specific effects on mast cell degranulation. DB was suspended in saline at 1 M prior to experiments and was diluted with saline to 2.5 mM.

Clinical symptoms were recorded every 10 min after the last OVA challenge and were used to derive clinical scores using the anaphylactic symptom scoring system developed previously.[15] Briefly, 0 indicated the absence of clinical symptoms; 1 indicated repetitive facial scratching; 2 indicated reduced activity and facial puffiness; 3 indicated periods of motionlessness for 1 min; 4 indicated no movement to gentle prodding; and 5 indicated death.

Diarrhea was assessed by visually monitoring the mice for up to 1 h following intragastric challenge. The mice were placed in individual cages and monitored for diarrhea symptoms for one hour. The feces appearance was scored as follows: 0, no change; 1, well formed; 2, soft, non-formed; 3, one episode of liquid diarrhea; and 4, at least two episodes of liquid diarrhea.[16] Finally, the mice were euthanized, and serum and the small intestine were collected.

Morphological analysis

Goblet cell mucins stained with periodic acid–Shiff and Alcian blue, pH 2.5 (PAS/AB) were used to estimate the amount of mucus accumulation, following a published protocol.[17] After sacrifice, the intestines were carefully dissected and fixed overnight in Carnoy's solution to preserve mucus. The jejunum was embedded in paraffin and processed for PAS/AB staining. From each animal, three sections were viewed with a Nikon microscope (using a 20× objective) with an attached digital camera.

Western blotting

Crude protein extracts were prepared as described previously,[18] and protein concentrations were determined using the bicinchoninic acid method. The proteins were resolved by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electro-transferred to nitrocellulose membranes before incubation with 2% bovine serum albumin (Sigma-Aldrich) blocking buffer for 1 h at room temperature to block nonspecific protein binding. Subsequently, the membranes were probed with antibodies against Lyn, PLCγ, p-Lyn, and p-PLCγ overnight at 4°C. After washing three times with Tris-buffered saline with Tween 20 (TBS-T), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Immunocomplexes were visualized by enhanced chemiluminescence detection (Bio-Rad, Hercules, CA, USA), followed by densitometric analysis of protein bands using the AlphaEase FC software.

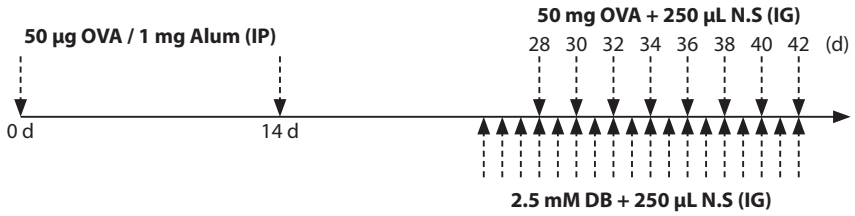

Figure 1. Treatment timeline for sensitization of mice. Animals received two OVA/alum intraperitoneal (i.p.) injections with subsequent intragastric (i.g.) treatment with OVA challenges every 2 days. To show the effects of DB on OVA-induced allergy, mice were treated intragastrically with 2.5 mM DB in 250 μL sterile saline every other day from day 25 to day 42.

RNA isolation and reverse transcription–quantitative polymerase chain reaction (RT-qPCR)

Total RNA was prepared from intestinal mast cells using an RNA extraction kit (TRIzol, Thermo Fisher Scientific) according to the manufacturer's protocol. RNA was converted into first-strand complementary DNA (cDNA) using a real-time reverse transcription kit (Cwbiotech, Beijing, China). qPCR was performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The primers used were: GAPDH (NM_017008.4) 5′-CTGGAGAAACCT GCCAAGTATG-3′ (sense) and 5′-GGTGGAAGAATGGGA GTTGCT-3′ (antisense); and rat FcεRIα (M21622) 5′-TGT GTA CTT GAA CGT GAT GCA A-3′ (sense) and 5′-TGT CTA AGA CCA C GT CAG CAG-3′ (antisense). The relative FcεRIα mRNA expression was calculated using GAPDH as the reference gene with the formula: $2^{-[Ct(Sample) - Ct(Gapdh)]}$.

Statistical analyses

Statistical comparisons between groups were performed using a one-way analysis of variance (ANOVA). The DB concentration was the independent variable and the mast cell mediators were the dependent variables. A p-value < 0.05 was considered to indicate a statistically significant difference. All results were obtained by evaluating the means ± standard deviation (SD).

Results

High DB concentrations are cytotoxic to mast cells

Previous oral toxicity studies indicated that DB has low acute and chronic toxicity in adult rats and mice,[19] but little is known about its toxicity to mast cells. For this reason, we exposed RBL-2H3 and KU812 cells to 0.01–100 mM DB and examined cell viability after 24 h. As shown in **Figure 2**, a DB concentration of up to 0.05 mM did not significantly show reduce viability.

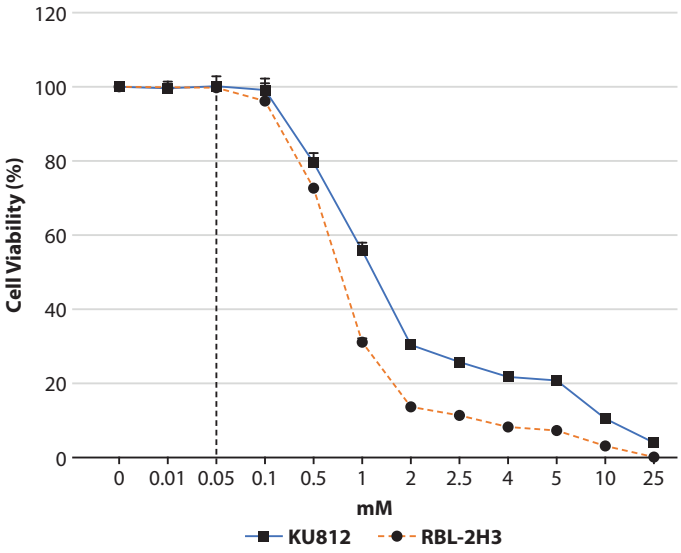

Figure 2. DB affects leukemia cell viability. Cell viability detected by CCK-8 assay in RBL-2H3 and KU812 cell lines treated for 24 h with the indicated doses of DB. The data shown are representative of 3 independent experiments (n = 5).

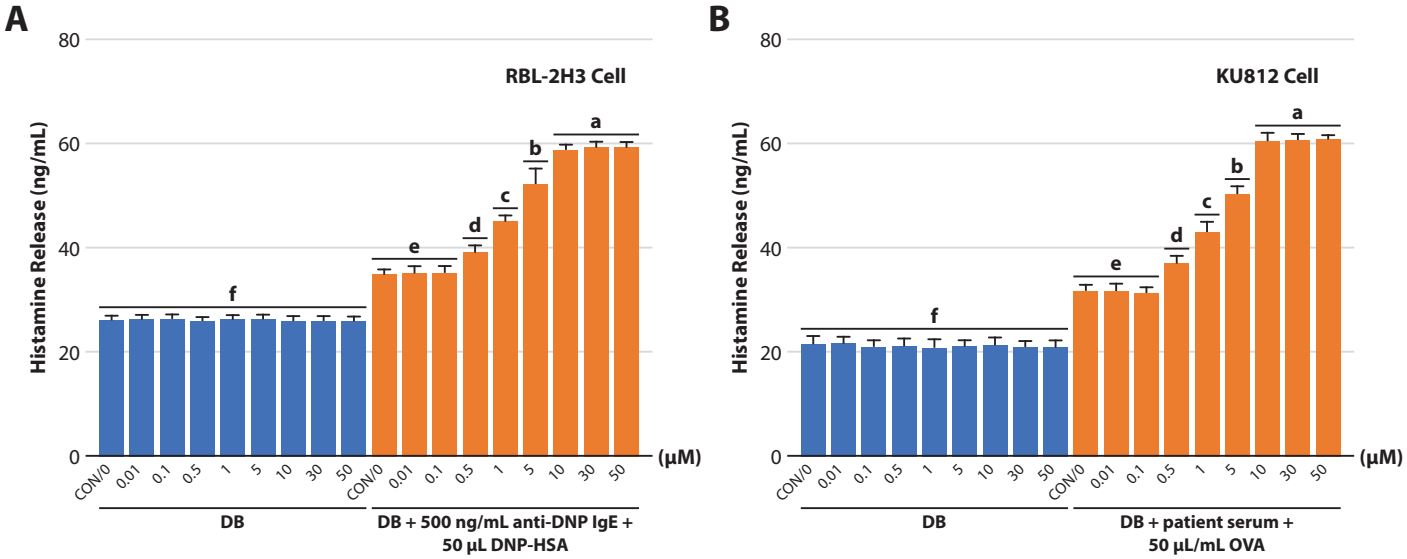

Figure 3. DB promotes histamine release IgE-mediated activation of RBL-2H3 and KU812 cells. (A) Cells were treated with the indicated doses of DB for 24 h, then the supernatants were collected and the rate of histamine release in supernatants in the cells. (B) Cells were treated with the indicated doses of DB for 24 h, meanwhile RBL-2H3 cells were sensitized overnight with 500 ng/mL anti-DNP IgE while KU812 cells were sensitized with 1:5 dilutions of patient serum. Then, cells were stimulated with DNP-HAS or OVA in Tyrode's buffer for 30 min at 37°C. The supernatants were collected and the rate of histamine release in supernatants in the cells. The data shown are representative of 3 independent experiments (n = 5). Different lowercase letters above the columns indicate significant difference between treatments for cells (p < 0.05). CON/0, the control group treated without DB.

Treatment of mast cells with DB increases histamine release in IgE-mediated activated RBL-2H3 and KU812 cells

Based on the findings using the CCK-8 assay, we selected non-cytotoxic doses of DB (≤ 0.05 mM) to evaluate the ability of this chemical to affect mast cell degranulation. As shown in **Figure 3**, treatment with 0.01–50 μM DB alone did not affect histamine release from non-IgE-activated cells. Nonetheless, DB generally exerted a dose-dependent increase in the level of histamine released from IgE-activated RBL-2H3/KU812 cells; this effect plateaued at 10 μM DB. Thus, we selected 10 μM DB as a non-toxic dose for the subsequent experiments.

DB promotes the expression of FcεRIα mRNA on mast cells

The cell surface levels of FcεRIα are critical for IgE-mediated mast cell functions. Therefore, we used immunofluorescence staining to determine whether DB affects the surface expression of FcεRIα. As illustrated in **Figure 4A and 4B**, treatment of IgE-activated cells with DB for 24 h significantly promoted the expression of FcεRIα in mast cells. We verified this finding with RT-qPCR: 10 μM DB increased FcεRIα mRNA expression by 29.4% in from RBL-2H3 cells and by 41.2% in KU812cells compared with IgE-activated cells without DB treatment. FcεRIα mRNA expression in cells treated with DB alone was not significantly different from the vehicle-treated cells, suggesting that DB alone does not significantly affect the expression of FcεRIα on mast cells.

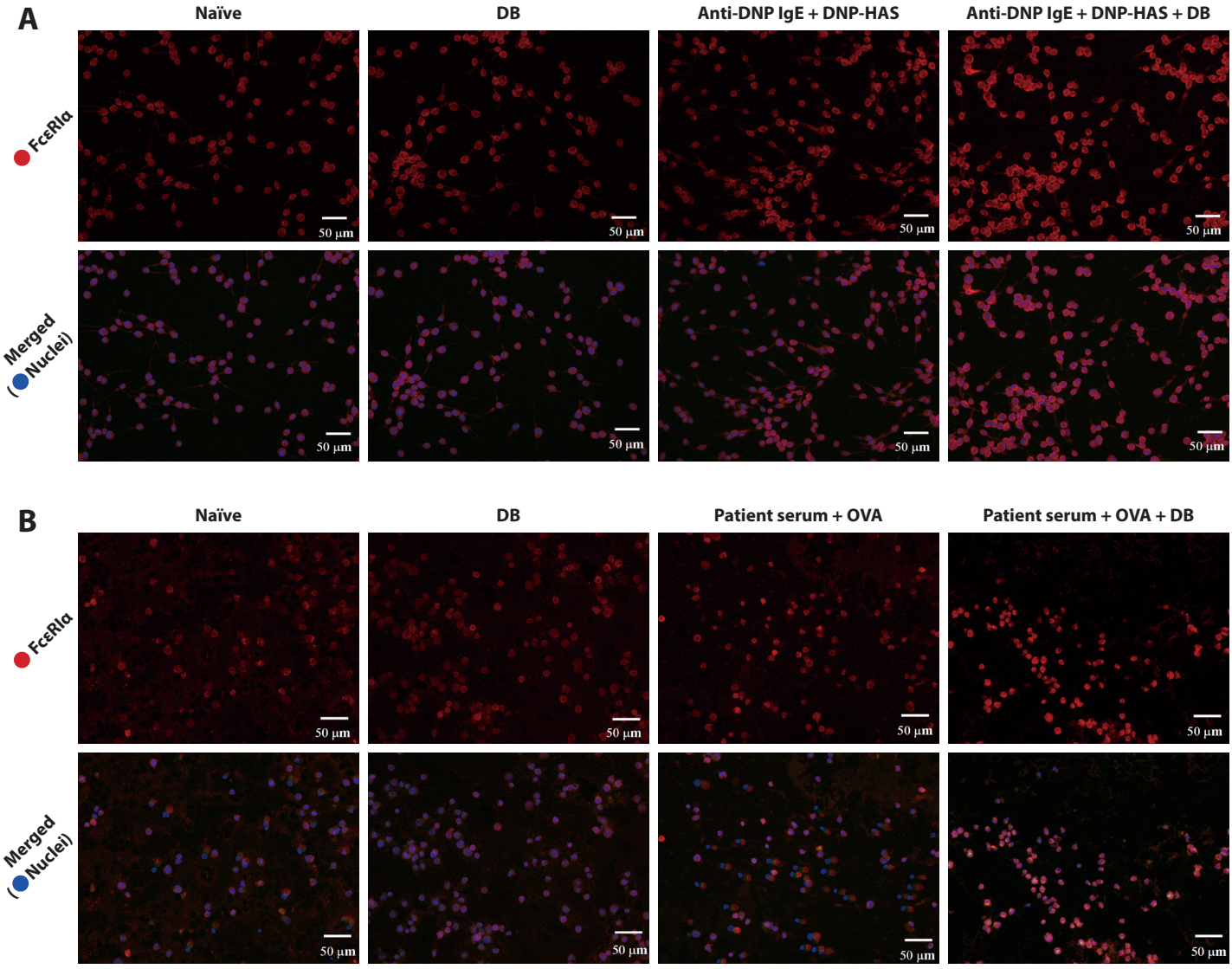

Figure 4. DB promotes FcεRIα expression in IgE-activated RBL-2H3 and KU812 cells. (A, B) Cells were treated with DB for 24 h, meanwhile incubated with IgE overnight. After 30-minute antigen challenge, cells were harvested to measure FcεRIα expression. Effects of DB on the expression of FcεRIα were measured by immunofluorescence in RBL-2H3 and KU812 cells, repectively (FcεRIα: red, DAPI: blue). (C) Expression levels of FcεRIα mRNA in RBL-2H3 and KU812 cells, respectively were measured by RT-qPCR and normalized to that of GAPDH reference mRNA. FcεRIα expression levels in each setting are shown relative to that in cells only treated with vehicle substances. The data shown are representative of 3 independent experiments (n = 4). $^{\#}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

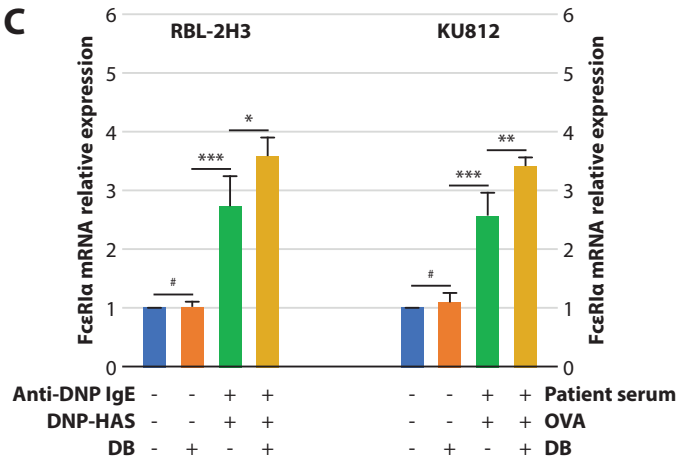

Figure 4. (Continued)

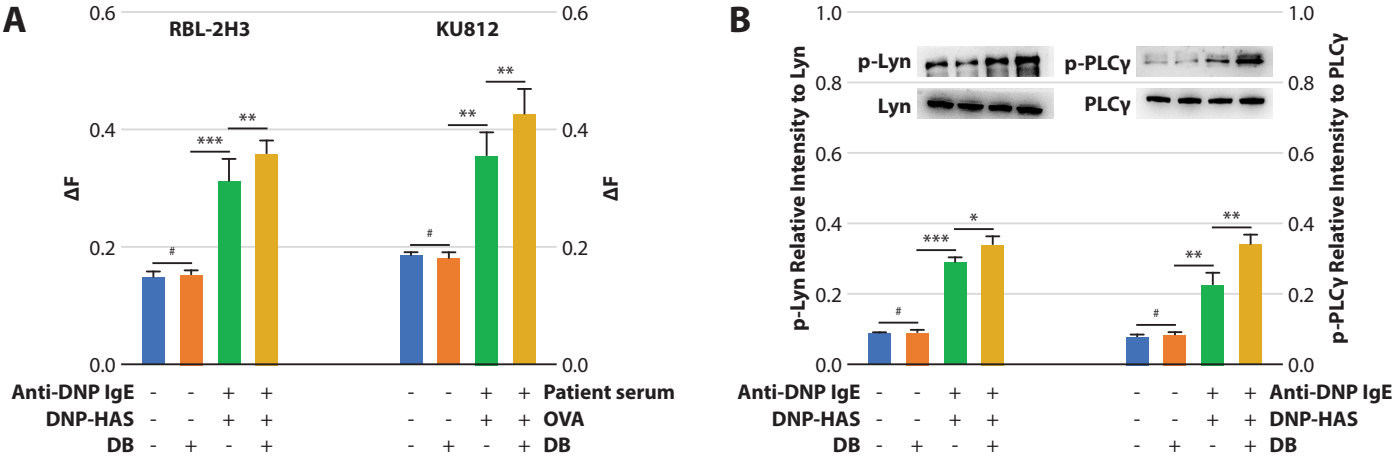

Figure 5. DB promotes Ca^{2+} mobilization and p-Lyn and p-PLCγ in IgE-mediated activation of mast cells. (A) After DB and IgE or human serum treatment, about 1 × 10^6 cells were pulsed with 5μM Fluo-3/AM containing 0.05% PluronicF127 for 30 min before addition of DNP–HAS or OVA and Ca^{2+} responses was measured as ΔF in 5 min. The data shown are representative of 3 independent experiments (n = 5). (B) Cells were treated with DB for 24 h, meanwhile incubated with IgE overnight. After 30-minute antigen challenge, cells were harvested and the expression levels of p-Lyn and p-PLCγ in RBL-2H3 cells were measured by western blotting. Antibodies against Lyn and PLC-γ were used as loading controls. The data shown are representative of 3 independent experiments (n = 3). $^{\#}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Treatment of mast cells with DB increases Ca^{2+} mobilization and p-Lyn and p-PLCγ levels in IgE-mediated activated cells

The binding of an allergen to IgE-FcεRI increases Ca^{2+} mobilization, followed by the degranulation of mast cells. In this context, we tested the ability of DB to affect IgE-activated Ca^{2+} influx in mast cells. As shown in **Figure 5A**, the amplitude of the intracellular Ca^{2+} level (shown as ΔF) in the IgE-activated group was obviously higher than in the naïve or DB-alone group, indicative of a significant degranulation response. As expected, treatment of IgE-activated cells with 10 μM DB resulted in a significant increase in ΔF compared with the IgE-activated cells without DB treatment: 0.281 ± 0.026 for RBL-2H3 cells and 0.328 ± 0.027 for KU812 cells. These data demonstrated that DB increases Ca^{2+} mobilization in IgE-activated cells, thereby affecting IgE-induced mast cell degranulation.

We also used western blotting to measure the changes in some important proteins in early FcεRI signaling, namely p-Lyn and p-PLCγ, in IgE-activated RBL-2H3 cells. DB significantly increased p-Lyn by 15% and p-PLCγ by 34% compared with the IgE-activated cells without DB treatment (**Figure 5B**).

Collectively, these data provide clear evidence that DB promotes IgE-mediated mast cell degranulation *in vitro* due to a change in FcεRIα expression, followed by increased Ca^{2+} influx and elevated p-Lyn and p-PLCγ levels, both of which regulate mast cell degranulation at the initial steps of FcεRI signaling.

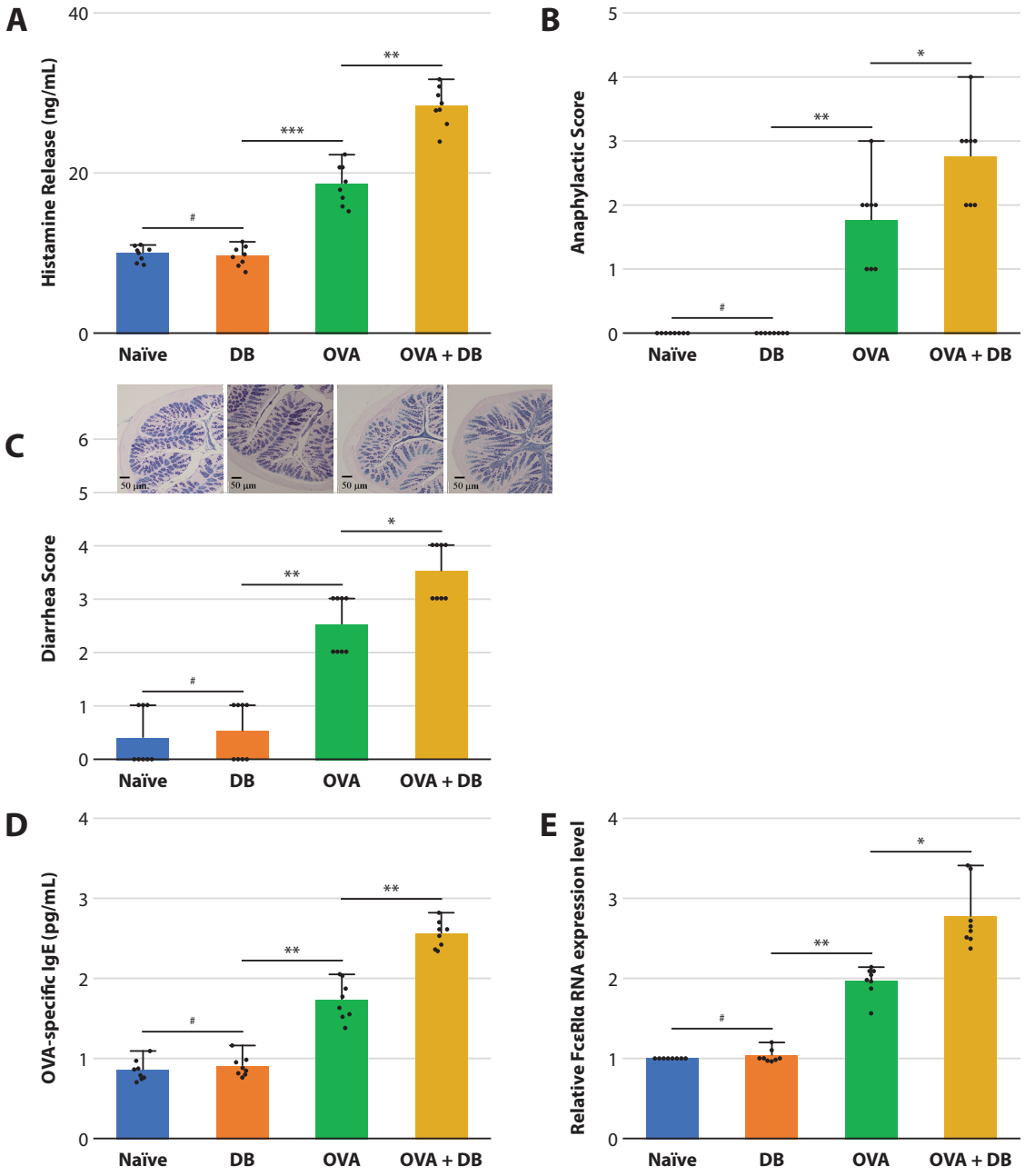

Figure 6. DB enhances OVA-induced BALB/c allergy and promotes FcεRIα expression in intestinal mucosal mast cells from OVA-induced allergy mice. (A) The effects of BD on the histamine releases from mice sera. (B) Peak anaphylactic symptom score of each individual mouse from all mice within 40 min after challenge. (C) The average clinical diarrhea scores at sacrifice and representative PAS/AB staining jejunum section from mice. (D) OVA-specific IgE in serum following saline or OVA challenges. (E) Effects of DB on FcεRIα mRNA expression in mast cells was measured by RT-qPCR. FcεRIα mRNA expression levels in each group are shown relative to that in naïve group mice. The data shown are representative of 3 independent experiments with 8 mice/group. $^{\#}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$.

DB promotes OVA-induced allergic diarrhea susceptibility in BALB/c mice and increases the FcεRIα mRNA expression in intestinal mucosal mast cells

Based on our *in vitro* findings, it was important to establish whether the effects of DB are relevant in the *in vivo* context. Thus, we utilized the well characterized OVA-induced BALB/c mouse model, which shows IgE-mediated allergic responses. We selected 2.5 mM DB to evaluate the ability of DB to affect mast cell degranulation *in vivo* because this dose did not produce a conditioned flavor aversion.[19,20] Moreover, we found that mice treated by intragastric gavage grew normally and did not display any clinical signs of DB-related toxicosis.

Similarly to the *in vitro* experiments, we randomly divided the mice into four groups: naïve (treated with only the vehicles), DB alone, control (OVA-challenged mice without DB treatment), and OVA and DB (OVA-challenged mice treated with DB). The DB alone group did not show any changes in histamine release (**Figure 6A**). Assessment of the anaphylactic responses revealed significant increases

in scores from the naïve group to the control group, with severe anaphylactic symptoms in the control group (**Figure 6B**). The OVA and DB group showed even more severe anaphylactic reactions, indicating that DB enhanced OVA-induced anaphylactic responses. The mice started developing acute diarrhea after three to four intragastric challenges. Compared with the control group, the OVA and DB group experienced significant diarrheic symptoms of food allergy (**Figure 6C**), accompanied by the accumulation of mucus in the jejunum, which was estimated based on the thickness of the mucus above the mucosa (**Figure 6C**, top panel). However, there were no noticeable changes involving the structural integrity of the intestines. In addition, serum OVA-specific IgE levels were elevated 2-fold in the OVA and DB group compared with the control group (**Figure 6D**). In addition, to analyze whether increased FcεRIα expression is required for the effects of DB on allergy in vivo, we examined FcεRIα mRNA expression mucosal mast cells isolated from the small intestines. FcεRIα mRNA expression was > 2-fold higher in the OVA and DB group compared with the control group (**Figure 6E**).

Discussion

Aside from its potential cytotoxicity to aquatic life, some researchers have reported physiological and pathophysiological effects of DB on mammals. Several reports related the use of DB to severe allergic reactions, including urticaria and asthma.[9–11] In the present study, we investigated the influence of DB on IgE-mediated mast cell degranulation and the underlying mechanisms involving FcεRIα, which is central to IgE-mediated mast cell function. Our analyses demonstrated that DB can promote IgE-mediated mast cell degranulation and OVA-induced allergic reactions, with DB responsible for increasing the cell-surface FcεRIα expression that is associated with an exacerbated mast cell response.

Besides FcεRI, there are several molecules that are important for the earliest steps of FcεRI signaling. For example, Lyn associates with FcεRI and initiates the associated signaling cascades.[21] PLCγ catalyzes the hydrolysis of phospholipids with the formation of diacylglycerol and inositol 1,4,5-triphosphate that is required for calcium mobilization.[22] Moreover, DB can activate largely nuclear and mitochondrial Ca^{2+} responses, resulting in a dramatically increased cytosolic Ca^{2+} response in cells.[23] We also found that DB elevated p-Lyn and p-PLCγ levels, as well as Ca^{2+} influx, following a change in FcεRIα expression in IgE-activated mast cells. These results suggest that DB enhances IgE-mediated mast cell activation at an early step of FcεRI signaling, initiated by increasing FcεRIα expression. Moreover, elevated FcεRIα expression is required to manifest the effects of DB on allergy *in vivo*. Specifically, DB enhanced the severity of OVA-induced anaphylactic and diarrheic symptoms, accompanied by thicker mucus in the jejunum and elevated histamine and OVA-specific IgE levels.

The available research shows that DB reaches concentrations of up to 400 ng L^{-1} in surface waters, which is considered a safe concentration for aquatic species, because the median lethal concentration for selected invertebrates and rainbow trout ranges from 400 to 1000 mg L^{-1}.[24] Notably, we found the half-maximal inhibitory concentration for RBL-2H3 cells was 0.306 mM (equivalent to 143 mg L^{-1}), and cytotoxic effects were still observed at 100 μM (equivalent to 46 mg L^{-1}). These levels are low compared with the median lethal concentration for invertebrates and fish, suggesting the need to re-evaluate the safety of DB at the current levels. Although detailed information about the annual global production of this compound is missing, its recently updated registration dossier reveals that approximately 100–1000 tons are manufactured and/or imported each year in the European Economic Area.[25] Equally concerning, the current evidence points toward a high environmental persistence of DB with no significant elimination once it is released into surface waters. Under favorable conditions, indirect photodegradation of DB can occur, but this process is considered to be very slow.[4] It seems that environmental DB levels will continue to increase and reach levels that adversely impact both aquatic species and humans. Therefore, it is imperative to closely watch the gradually increasing concentrations of DB and its biodegradation products, and to provide better assessment of the potential risks.

In the present study, we showed that exposure to non-toxic doses of DB (1–100 μM) upregulated the IgE-mediated degranulation response in mast cells *in vivo* and *in vitro* by upregulating FcεRIα expression. This provides clear mechanistic evidence that allergy susceptibility is associated with exposure to DB. Intriguingly, it was reported that 300 μM and 1000 μM DB inhibited IgE-dependent activation of cord blood–derived mast cells and the human mast cell line HMC-1.2.[26] However, we found that such high levels of DB were cytotoxic, and we were careful to exclude these effects in our experiments. Moreover, recent studies in the context of cancer showed that the viability of primary human acute myeloid leukemia leukemic cells was reduced after exposure to > 50 μM DB,[27] but this concentration was not cytotoxic in our study. It is possible that differences in cell lines or drug preparations could explain these discrepancies. Nonetheless, it is worth noting that the European Union requires a minimum concentration of 10 mg L^{-1} (21 μM) DB as a denaturing agent in applicable products.[28] In view of our results, where 1–100 μM DB can enhance mast cell degranulation, this concentration clearly poses potential allergy risks.

Conclusion

In conclusion, our results indicate that DB promotes IgE-mediated mast cell degranulation and OVA-induced allergic diarrhea in BALB/c mice, demonstrating the allergy susceptibility associated with exposure to DB. DB is a known agonist of T2Rs, binding to them and activating the associated signaling cascade. Additional work will explore whether increased DB levels enhance IgE-mediated allergy by activating the ILC2–Th2 circuit via T2Rs on tuft cells, which initiate type 2 immunity in response to various luminal signals.[29]

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Competing interests

The authors declare no competing interests.

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