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FFA2 Activation Ameliorates 2,4-Dinitrochlorobenzene-Induced Atopic Dermatitis in Mice

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Abstract

Gut microbiota produce dietary metabolites such as short-chain fatty acids, which exhibit anti-inflammatory effects. Free fatty acid receptor 2 (FFA2, formerly known as GPR43) is a specific receptor for short-chain fatty acids, such as acetate that regulates inflammatory responses. However, the therapeutic potential of FFA2 agonists for treatment of atopic dermatitis has not been investigated. We investigated the efficacy of the FFA2 agonist, 4-chloro-α-(1-methylethyl)-*N*-2-thiazoylylbenzeneacetanilide (4-CMTB), for treatment of atopic dermatitis induced by 2,4-dinitrochlorobenzene (DNCB). Long-term application of DNCB to the ears of mice resulted in significantly increased IgE in the serum, and induced atopic dermatitis-like skin lesions, characterized by mast cell accumulation and skin tissue hypertrophy. Treatment with 4-CMTB (10 mg/kg, i.p.) significantly suppressed DNCB-induced changes in IgE levels, ear skin hypertrophy, and mast cell accumulation. Treatment with 4-CMTB reduced DNCB-induced increases in Th2 cytokine (IL-4 and IL-13) levels in the ears, but did not alter Th1 or Th17 cytokine (IFN-γ and IL-17) levels. Furthermore, 4-CMTB blocked DNCB-induced lymph node enlargement. In conclusion, activation of FFA2 ameliorated DNCB-induced atopic dermatitis, which suggested that FFA2 is a therapeutic target for atopic dermatitis.

Key Words: Atopy, Dermatitis, Free fatty acid receptor 2, FFA2, Short chain fatty acids

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disorder characterized by intense pruritus and eczematous patches or plaques with a complex etiology (Davidson *et al*., 2019). Atopic dermatitis affects up to 20% of children worldwide and can significantly impair the quality of life owing to emotional distress, sleep disruption, and social awkwardness (Leung and Guttman-Yassky, 2017). Gut microbiota produce dietary metabolites such as short-chain fatty acids, which exert anti-inflammatory effects (Trompette *et al*., 2014). Free fatty acid receptor 2 (FFA2, formerly known as GPR43) is a specific receptor for short-chain fatty acids such as acetate, propionate, and butyrate (Brown *et al*., 2003; Le Poul *et al*., 2003; Nilsson *et al*., 2003). Activation of FFA2 by short-chain fatty acids regulates inflammatory responses (Ulven, 2012; Miyamoto *et al*., 2017; Sun *et al*., 2017; Tan *et al*., 2017). Furthermore, FFA2 knockout mice did not experience resolution of certain inflammatory responses in models of colitis, arthritis, and asthma, which indicated that FFA2 was necessary for resolution of inflammation (Maslowski *et al*., 2009). Lower concentrations of fecal acetate at 3 months of age correlated with an increased risk for the development of atopy in childhood (Arrieta *et al*., 2015). Although accumulating evidence supports the existence of a metabolite-mediated gut-skin axis of communication, the therapeutic potential of FFA2 agonists has not been investigated for treatment of atopic dermatitis (Lee *et al*., 2018). Therefore, we investigated the efficacy of the FFA2 agonist, 4-chloro-α-(1-methylethyl)-*N*-2-thiazoylylbenzeneacetanilide (4-CMTB), for treatment of atopic dermatitis induced by 2,4-dinitrochlorobenzene (DNCB) in mice (Lee *et al*., 2008; Smith *et al*., 2011).

MATERIALS AND METHODS

Materials

4-Chloro-α-(1-methylethyl)-*N*-2-thiazoylylbenzeneace-

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tanilide was purchased from Tocris (Ellisiville, MO, USA). Acetone was purchased from Junsei Chemical (Tokyo, Japan). 2,4-Dinitrochlorobenzene and olive oil were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Seven-week-old male Balb/c mice were purchased from Daehan Biolink (DBL; Seoul, Korea) and housed in the laboratory animal facility at Pusan National University. The mice were housed three per cage in standard plastic cages with sawdust as bedding, and maintained at 22-24°C, with humidity at 60 ± 5%, with alternating light/dark cycles (lights were on between 7:00 h and 19:00 h), and provided with standard laboratory chow and water ad libitum (Huang *et al*., 2018). The animal experiment protocol for this study was reviewed and approved by the Pusan National University–Institutional Animal Care Committee (PNU–IACUC) (Approval Number PNU-2019-2219).

Induction of atopic dermatitis in Balb/c mice and 4-CMTB administration

Following a simple randomization procedure, eight-week-old male Balb/c mice were divided into the following three groups (n=5 each): a vehicle (acetone:olive oil, 3:1)-treated control group, a DNCB-treated group, and a 4-CMTB/DNCB-treated group. To induce experimental atopic dermatitis, the ventral skin was shaved, and 300 μL of 1% DNCB in acetone:olive oil (3:1) was applied to the ventral skin on day 0. Starting on day 7, the mice were challenged with 200 μL of 0.3% DNCB applied to the ears every other day for up to 48 days. From day 19 until completion of the study, the 4-CMTB/DNCB-treated group was administrated 4-CMTB (10 mg/kg body weight) via intraperitoneal injection 30 min prior to challenge. All mice were sacrificed on day 49.

Histologic analysis and mast cell count in the skin

After sacrifice on day 49, ear sections from mice in the different experimental groups were obtained and examined. Briefly, the ears were fixed in 10% formalin, dehydrated in 30% sucrose solution, and embedded in O.C.T. compound. Sections (8 μm) were stained with toluidine blue O (cat. T3260, Sigma-Aldrich) to visualize mast cells in the skin, and with hematoxylin and eosin (H&E) to visualize immune cell infiltration. Ear tissues from each of the five mice in each group were examined. For toluidine blue O staining, O.C.T. compound was removed from the sections, and the sections were hydrated and stained with toluidine blue O reagent for 2 min. Following staining, the sections were rinsed, dehydrated, and mounted on coverslips. Numbers of mast cells were counted from the photographs of toluidine blue O staining. For H&E staining, O.C.T. compound was removed from the sections, and the sections were hydrated and stained with hematoxylin reagent for 15 s. After rinsing with warm tap water, the sections were stained with eosin reagent for 10 s, rinsed, dehydrated, and then mounted on coverslips (Park and Im, 2019b).

Measurement of total serum IgE levels

Immunoglobulin E (IgE) levels in serum isolated from each group were measured using enzyme-linked immunosorbent assay (ELISA) kits. Briefly, 96-well plates (NUNC, Penfield, NY, USA) were coated with a capture antibody specific for IgE (cat. 88-50460-88, eBioscience, San Diego, CA, USA) and incubated overnight at 4°C. After washing, the plates were incubated with a blocking buffer for 2 h at room temperature. Serial dilutions of standard IgE were prepared and added to the appropriate wells to generate a calibration curve. Serum samples were added to the appropriate wells. The plates were incubated for 2 h at room temperature on a shaker, and then washed two times. A biotinylated detection antibody specific for IgE (cat. 88–50460–88, eBioscience) was added to each well, and the plates were incubated for 1 h at room temperature on a shaker. The plates were washed 4 times, and avidin-horseradish peroxidase (HRP) was added to each well. The plates were then incubated at room temperature for 30 min on a shaker. The plates were then washed 4 times and incubated with substrate solution at room temperature for 15 min. Stop solution was added, and the absorbance was measured at 450 nm.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The expression levels of inflammatory markers in the ears of mice were measured using RT-PCR. First-strand cDNA was synthesized from total RNA isolated from ear tissue using Trizol reagent (Invitrogen, Waltham, MA, USA). Synthesized cDNA products, primers for each gene, and Promega Go-Taq DNA polymerase (Madison, WI, USA) were used for PCR analysis. Specific primers and PCR conditions were as previously described (Park and Im, 2019a). Aliquots (7 μL) were electrophoresed in 1.2% agarose gels and stained with StaySafe™ Nucleic Acid Gel Stain (Real Biotech Corporation, Taipei, Taiwan). The intensity of each PCR product was quantified by using ImageJ software (NIH, Bethesda, MD, USA) and normalized to GAPDH levels (Lee *et al*., 2017).

Statistics

Results are expressed as the mean ± standard error of the

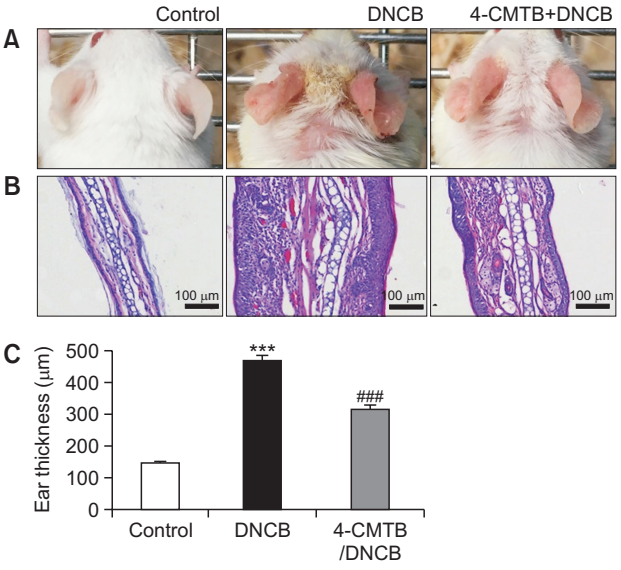

Fig. 1. Effect of 4-CMTB on DNCB-induced atopic dermatitis in ears. (A) Macroscopic view of the ears. (B) H&E staining of ear tissue sections. (C) Ear thickness was measured from the sections (n=5). Statistically significant at ***p<0.001 level vs. the vehicle-treated mice and at $^{###}p$<0.001 level vs. the DNCB-treated mice.

mean (SEM) of 5 determinations for each animal experiment. Statistical significance was evaluated using analysis of variance (ANOVA) and Tukey's multiple comparison test. Differences were considered statistically significant for p values <0.05. Analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

4-CMTB suppressed DNCB-induced atopic dermatitis in the ears

To investigate the role of FFA2 in atopic dermatitis, we generated a DNCB-induced atopic dermatitis model using Balb/c mice. The mice were then treated with 4-CMTB, an FFA2 agonist, during the elicitation phase to evaluate the potential of FFA2 as a target for treatment of atopic dermatitis. Mice were treated with 0.3% DNCB on both ears every other day for 42 days to induce atopic dermatitis. 4-CMTB (10 mg/kg) was injected intraperitoneally 30 min prior to DNCB challenge starting on Day 19. Symptoms of DNCB-induced atopic dermatitis were by edema, erythema, and cracking of the skin in the exposed area (Fig. 1A). These symptoms were reduced in the group treated with 4-CMTB (Fig. 1A). In addition, H&E staining confirmed that DNCB induced increased infiltration of immune cells compared to the control group (Fig. 1B). Furthermore, the epidermis was obviously thickened upon visual inspection due to hyperkeratosis (Fig. 1B). 4-CMTB treatment significantly suppressed DNCB-induced hypertrophy of the epidermis and reduced DNCB-induced immune cell infiltration to the ears (Fig. 1B, 1C).

4-CMTB suppressed DNCB-induced infiltration of mast cells in the ears

Toluidine blue O staining was used to measure infiltration of mast cells into the dermis. Mast cells appeared as small, red-purple dots (Fig. 2A). Increased infiltration of mast cells and hypertrophy were observed in the DNCB-treated group (Fig. 2A). In contrast, fewer stained cells were observed in sections from mice treated with DNCB plus 4-CMTB than in sections from mice in the group treated only with DNCB (Fig. 2A). Mast cell number was semi-quantitatively analyzed. As shown in Fig. 2B, the number of mast cells in the dermis was higher in the DNCB group than in the control group. 4-CMTB treatment significantly reduced DNCB-induced mast cell infiltration into the dermis (Fig. 2B).

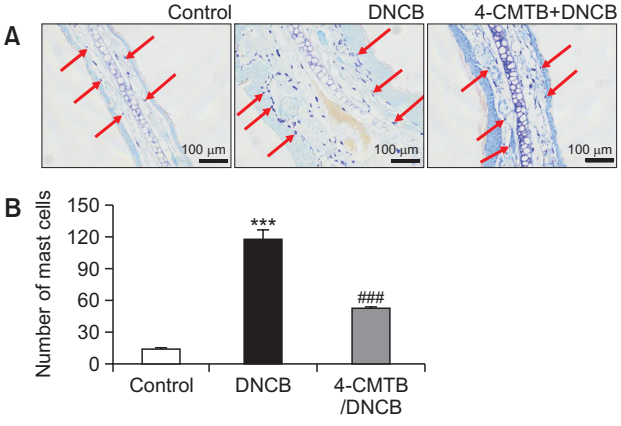

Fig. 2. Effect of 4-CMTB on DNCB-induced mas cell accumulation in ears. (A) Toluidine blue O staining of ear samples. Red arrows indicate several mast cells. (B) Number of mast cells. Results are presented as means ± SEM (n=5). Statistically significant at ***p<0.001 level vs. the vehicle-treated mice and at ###p<0.001 level vs. the DNCB-treated mice.

4-CMTB suppressed DNCB-induced increases in serum IgE levels

Serum IgE levels were measured to determine the immunological effects of DNCB and 4-CMTB. Hyper-production of IgE was observed in the sera of DNCB-treated mice, and 4-CMTB treatment significantly suppressed this increase (Fig. 3).

4-CMTB suppressed DNCB-induced increases in cytokine levels in the ears

Atopic dermatitis is believed to be regulated by the Th2, Th1, and Th17 responses (Koga *et al.*, 2008; Kim *et al.*, 2014; Muraro *et al.*, 2016). Thus, the Th2, Th17, and Th1 responses were investigated by measuring levels of IL-4, IL-13, IL-17A, and INF-γ in the ears. Messenger RNA levels of each of the cytokines evaluated were significantly increased in ear tissue following application of DNCB (Fig. 4). 4-CMTB significantly reduced DNCB-induced increases in the Th2 cytokines IL-4 and IL-13 (Fig. 4A, 4B). In contrast, the levels of the Th17 and Th1 cytokines IL-17A and INF-γ were not significantly reduced by 4-CMTB treatment (Fig. 4C, 4D).

4-CMTB suppressed DNCB-induced atopic responses in lymph nodes

We evaluated lymph node size in drained lymph nodes. Lymph nodes are important components of the lymphatic system, and are filled with B cells and T cells. When an infection or an allergic reaction occurs, lymph nodes undergo swelling. The lymph nodes of mice treated with DNCB were swollen compared to those of the control mice, and lymph node weight was increased by 877% (Fig. 5). 4-CMTB significantly suppressed the DNCB-induced increase in lymph node weight by 43% (Fig. 5).

DISCUSSION

In this study, we showed that FFA2 activation could ameliorate the symptoms of atopic dermatitis induced by treatment of DNCB in mice, as evidenced by reduction of atopic skin

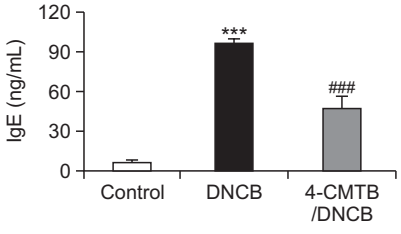

Fig. 3. Effect of 4-CMTB on DNCB-induced increases in serum IgE levels. Serum IgE levels. Results are presented as means ± SEM (n=5). Statistically significant at ***p<0.001 level vs. the vehicle-treated mice and at ###p<0.001 level vs. the DNCB-treated mice.

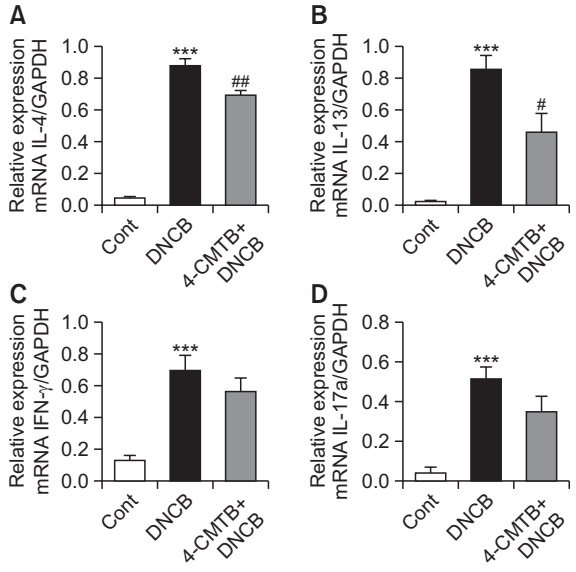

Fig. 4. Effect of 4-CMTB on DNCB-induced increases in cytokine levels in ears. RT-PCR analyses for Th2 cytokines (IL-4 and IL-13), a Th17 cytokine (IL-17A), and a Th1 cytokine (INF-γ). Results are presented as means ± SEM (n=5). Statistically significant at ***p<0.001 level vs. the vehicle-treated mice and at $^{\#}p$<0.05, $^{\#\#}p$<0.01 level vs. the DNCB-treated mice.

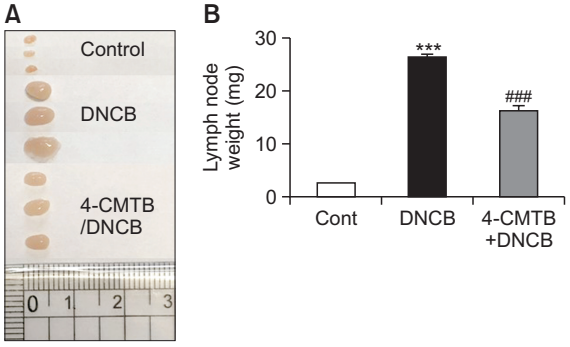

Fig. 5. Effect of 4-CMTB on DNCB-induced atopic dermatitis in lymph nodes. (A) Photographs of lymph nodes. (B) Weights of lymph nodes. Statistically significant at ***p<0.001 level vs. the vehicle-treated mice and at $^{\#\#\#}p$<0.001 level vs. the DNCB-treated mice.

lesions, reduced hypertrophy in the epidermis, suppression of mast cell infiltration into the dermis, suppression of serum IgE levels, reduced Th2 cytokine levels, and reduced lymph node swelling. These observations supported three main conclusions. First, FFA2 receptors functioned to regulate the immune response in the dermis. Second, an FFA2 agonist suppressed the Th2 immune response in a model of atopic dermatitis. Third, systemic administration of 4-CMTB resolved atopic dermatitis in a mouse model.

Daily intake of fiber-rich foods supplies resources for the gut microbiota to produce short chain fatty acids. Activation of FFA2 by short-chain fatty acids could lead to regulation of inflammatory responses in skin diseases. A previous study showed that lower concentrations of fecal acetate at 3 months of age correlated with increased risk for development of atopy in childhood (Arrieta *et al*., 2015). Similarly, mice fed a high-fiber diet had increased circulating levels of short-chain fatty acids and were protected against allergic inflammation in the lung (Trompette *et al*., 2014).

Short-chain fatty acids as dietary metabolites produced by gut microbiota have been shown to exert anti-inflammatory effects and to regulate inflammatory responses through FFA2 (Ulven, 2012; Trompette *et al*., 2014; Miyamoto *et al*., 2017; Sun *et al*., 2017; Tan *et al*., 2017). Our study showed that FFA2 can act as an immune regulator in the gut-skin axis, and that this receptor may be a promising target for treatment of atopic dermatitis. A previous study showed that stimulation of FFA2 by short-chain fatty acids was necessary for normal resolution of several inflammatory responses in disease models of colitis, arthritis, and asthma (Maslowski *et al*., 2009). Our study showed that FFA2 also mediated resolution of the inflammatory skin disease atopic dermatitis in a mouse model.

Activation of FFA2 may have suppressed atopic dermatitis through several mechanisms. FFA2 in bone marrow-derived cells was shown to be largely responsible for inflammatory responses (Maslowski *et al*., 2009). Furthermore, FFA2 expression was reported in neutrophils and eosinophils in mice and humans (Maslowski *et al*., 2009; Theiler *et al*., 2019). Acetate induced robust calcium influx and chemotaxis in mouse and human neutrophils and eosinophils through FFA2 activation (Maslowski *et al*., 2009; Theiler *et al*., 2019). Therefore, FFA2 activation in immune cells may have contributed to reduced atopic response in our study, as evidenced by reduced IgE levels, cytokine levels, and lymph node size in mice treated with the FFA2 agonist 4-CMTB.

In this study, 4-CMTB was administrated by intraperitoneal injection instead of oral or topical administration. Because 4-CMTB was newly available commercially and has been applied only once in an *in vivo* experiment intraperitoneally (Schofield *et al*., 2018), we applied 4-CMTB by intraperitoneal injection. Intraperitoneal injection was a safe choice to see the direct pharmacodynamics effect of 4-CMTB without interruption of pharmacokinetic factors such as poor absorption. Because the purpose of the study was to evaluate FFA2 as a therapeutic target for atopic dermatitis, the administration route did not matter for it. Topical application of 4-CMTB was also considered. However, in order to get immune suppressive effect of 4-CMTB on FFA2 in immune cells as like acetate, we thought that plasma concentration of 4-CMTB should be high enough in blood and/or lymphatic circulations. Therefore, we did not apply 4-CMTB on the skin topically. Initially, 15 mg/kg of 4-CMTB was chosen, because the previous *in vivo* study used that dose (Schofield *et al*., 2018). However, the repeated administration of 15 mg/kg 4-CMTB was not tolerated by mice in this model. Therefore, we reduced the dose to 10 mg/kg and it was tolerated in mice, similar effects were observed with 5 mg/kg dose (data not shown).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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