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Transient receptor potential ankyrin 1 activation enhances hapten sensitization in a T-helper type 2-driven fluorescein isothiocyanate-induced contact hypersensitivity mouse model

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ABSTRACT

Some chemicals contribute to the development of allergies by increasing the immunogenicity of other allergens. We have demonstrated that several phthalate esters, including dibutyl phthalate (DBP), enhance skin sensitization to fluorescein isothiocyanate (FITC) in a mouse contact hypersensitivity model, in which the T-helper type 2 (Th2) response is essential. On the other hand, some phthalate esters were found to activate transient receptor potential ankyrin 1 (TRPA1) cation channels on sensory neurons. We then found a positive correlation between the enhancing effects of several types of phthalate esters on skin sensitization to FITC and their ability to activate TRPA1. Here we examined the involvement of TRPA1 in sensitization to FITC by using TRPA1 agonists other than phthalate esters. During skin sensitization to FITC, the TRPA1 agonists (menthol, carvacrol, cinnamaldehyde and DBP) augmented the ear-swelling response as well as trafficking of FITC-presenting dendritic cells to draining lymph nodes. We confirmed that these TRPA1 agonists induced calcium influx into TRPA1-expressing Chinese hamster ovary (CHO) cells. We also found that TRPA1 antagonist HC-030031 inhibited DBP-induced calcium influx into TRPA1-expressing CHO cells. After pretreatment with this antagonist upon skin sensitization to FITC, the enhancing effect of DBP on sensitization was suppressed. These results suggest that TRPA1 activation will become a useful marker to find chemicals that facilitate sensitization in combination with other immunogenic haptens.

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Introduction

Studies on chemicals that facilitate sensitization to allergens or haptens have been drawing much attention. As a mechanism of such an enhancing effect on the sensitization process, the roles of neurons that detect noxious substances have been recognized. For example, sensory neurons that detect noxious substances are proposed to be involved in the initiation of immediate type allergies, in which immune responses are polarized toward T-helper type 2 (Th2) (Palm et al., 2012). Experimentally, the fluorescein isothiocyanate (FITC)-induced skin sensitization mouse model is known to have the feature that the Th2 response is essential during sensitization (Dearman and Kimber, 2000; Maruyama et al., 2007b; Takeshita et al., 2004). We have demonstrated that several phthalate esters, including dibutyl phthalate (DBP), enhance skin sensitization to FITC

(Imai et al., 2006; Sato et al., 1998). Epidemiological studies have also revealed an association between phthalate esters in house dust and allergic diseases among children (Kolarik et al., 2008).

As a nociceptive receptor, transient receptor potential ankyrin 1 (TRPA1) has been recognized (Story et al., 2003). This molecule is a member of the TRP family of calcium permeable cation channels that are expressed on sensory neurons. These channels can be activated through covalent modification of specific cysteine residues located within their cytoplasmic N-terminal domains (Macpherson et al., 2007). This unique mechanism allows TRPA1 to be activated by various structurally distinct chemicals that have electrophilic properties. Chemicals and skin allergens that bind to cysteine may potentially activate TRPA1, but membrane permeability is required due to the intracellular location of the critical cysteines. Electrophilic TRPA1 agonists include plant-derived allylic mono-, di-, and trisulfides (Bautista et al., 2005), isothiocyanates such as allyl isothiocyanate (AITC) (Bandell et al., 2004; Jordt et al., 2004), and α , β -unsaturated aldehydes such as cinnamaldehyde (CNA) (Bandell et al., 2004). In addition, some non-electrophilic chemicals can also activate TRPA1. Non-electrophilic TRPA1 agonists include menthol (Karashima et al., 2007), carvacrol (Xu et al., 2006), thymol (Lee et al., 2008), and Δ^9 -tetrahydrocannabinol (Jordt et al., 2004).

Abbreviations: AITC, allyl isothiocyanate; CHO, Chinese hamster ovary; CHS, contact hypersensitivity; CGRP, calcitonin gene-related peptide; CNA, cinnamaldehyde; DBP, dibutyl phthalate; DC, dendritic cell; FITC, fluorescein isothiocyanate; TRPA1, transient receptor potential ankyrin 1; TRPV, transient receptor potential vanilloid.

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We have directly demonstrated that dibutyl phthalate (DBP) activates TRPA1 on AITC-responsive neurons isolated from mouse dorsal root ganglia as well as that on TRPA1-expressing Chinese hamster ovary (CHO) cells (Shiba et al., 2009). In addition, there is a positive relationship between the enhancing activity toward skin sensitization to FITC and the ability of TRPA1 activation among various phthalate esters (Imai et al., 2006; Shiba et al., 2009). These results suggest that DBP and some other phthalate esters may exert enhancing effects through TRPA1 activation. As a mechanism in the skin sensitization process, trafficking of hapten-presenting dendritic cells (DCs) from the skin to draining lymph nodes is important. We have also found that DC-trafficking is promoted by phthalate esters that exhibit enhancing effects on sensitization as well as TRPA1 activation (Imai et al., 2006).

One of the unsolved questions is whether or not other known TRPA1 agonists have enhancing effects on FITC-induced skin sensitization and on DC trafficking. In addition, a TRPA1-selective antagonist was tested as to its effect on DBP-induced TRPA1 activation *in vitro*. Finally, we examined whether or not *in vivo* treatment with this antagonist could inhibit the enhancing effect of DBP on sensitization to FITC.

Materials and methods

Chemicals and antibodies

Acetone, DBP, (–)-menthol, (+)-camphor, AITC, kanamycin sulfate and a 0.5% (w/v) methylcellulose 400 sterile solution were purchased from Wako Pure Chemicals (Osaka, Japan), FITC, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and Fluo-4AM from Dojindo Laboratories (Kumamoto, Japan), dimethylsulfoxide (DMSO) from Nacalai Tesque (Kyoto, Japan), and ionomycin, blasticidin, zeocin and tetracycline from Life Technologies (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceuticals (Tokyo, Japan), Ham's F-12 medium, Hanks' balanced salt solution (HBSS), bovine serum albumin (BSA; fraction V), CNA, carvacrol, probenecid and poly-L-lysine from Sigma-Aldrich (St Louis, MO), fetal bovine serum (FBS) from Hyclone (South Logan, UT), and 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-*N*-(4-isopropylphenyl) acetamide (HC-030031) from ENZO Life Sciences (Farmingdale, NY). Phycoerythrin (PE)-conjugated hamster anti-CD11c monoclonal antibodies (mAb) (clone HL3; IgG1) and a PE-conjugated hamster IgG1 isotype control were purchased from BD Biosciences (San Jose, CA).

Animals

Female BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan) at 7 weeks of age and held for 1 week before use. They were housed at 22 to 24 °C with 50 to 60% humidity under artificial lighting conditions with a 12-h light/dark cycle. They had access to food (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*. Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka, and animal studies were reviewed and approved by the University of Shizuoka Institutional Animal Care and Use Committee.

Cells

CHO cells stably expressing TRPA1 (TRPA1-CHO cells) were established with a T-Rex system (Life Technologies) as described previously (Shiba et al., 2009). As a control, T-Rex CHO cells, which stably express the tetracycline repressor protein, were used. Cells were maintained in Ham's F-12/DMEM (1:1) containing 10% FBS, 60 µg/ml kanamycin, 5 µg/ml blasticidin and 200 µg/ml zeocin at 37 °C under a humidified atmosphere of 5% CO₂/95% air.

Measurement of TRPA1 activation *in vitro*

The ligand-induced change in the intracellular calcium level was measured with a fluorescent calcium indicator, Fluo-4 (Shiba et al., 2009). The measurements were carried out automatically using a fluorometric imaging plate reader, FLEXstation II (Molecular Devices, Sunnyvale, CA). TRPA1-CHO cells were cultured in the poly-L-lysine-coated wells of a 96-well, black-walled, clear-bottomed plate (Corning #3340; Corning, NY) at a density of 4.0×10^4 cells/well for 24 h at 37 °C in the presence of 1 µg/ml tetracycline to induce TRPA1. The cells were then loaded with 3 µM Fluo-4AM in HBSS containing 20 mM HEPES (pH 7.4), 1 mM CaCl₂, 0.1% BSA and 2.5 mM probenecid (loading buffer) for 1 h at 37 °C. After the wells had been washed to remove Fluo-4AM, each well received the loading buffer. As a selective antagonist of TRPA1, HC-030031 was used. Ligand samples with or without the inhibitor were automatically added to appropriate wells. To record maximal responses, 5 µM ionomycin was added. Stock solutions of samples and the inhibitor were prepared in DMSO. The stock solutions were diluted on the day of the experiment. The final concentration of DMSO did not exceed 0.1%.

FITC-induced CHS

Experiments were performed as described previously (Imai et al., 2006). On days 0 and 7, 160 µl of an FITC solution (0.5% [w/v]) in one of the following solvents was epicutaneously applied to the shaved forelimb skin of a mouse under anesthesia. The solvents were acetone alone, DBP (2 and 50% [v/v] in acetone), menthol (1, 10 and 50% [w/w] in acetone), carvacrol (5% [v/v] in acetone), CNA (20% [v/v] in acetone), and camphor (20% [w/w] in acetone). We chose the concentrations of carvacrol and CNA as high as possible considering *in vivo* toxicity. On day 14, the ear thickness baseline (0 h) in each animal was determined using a dial thickness gauge (Mitsutoyo, Kanagawa, Japan). Mice were challenged by applying 20 µl of an FITC solution (0.5% [w/v] in acetone/DBP [1:1]) on the right auricle, and then the ear thickness was measured after 24, 48 and 72 h. The left auricle was treated with 20 µl of acetone/DBP alone as a control. Ear swelling at X h is defined as follows: [(ear thickness of the right ear at X h) – (ear thickness of the right ear at 0 h)] – [(ear thickness of the left ear at X h) – (ear thickness of the left ear at 0 h)]. In some experiments, mice were pretreated with a selective TRPA1 antagonist, HC-030031, which was suspended (20 mg/ml) in 0.5% methylcellulose (Bessac et al., 2009). Mice received 200 µl of the HC-030031 suspension (4 mg/mouse) or the vehicle alone by ip injection 1 h before sensitization on days 0 and 7. Each experiment was performed with 7–11 animals per group. The dose of HC-030031 was selected according to the reports regarding mice (Bessac et al., 2009; Caceres et al., 2009).

DC trafficking

Experiments were performed as described previously with some modifications (Imai et al., 2006; Maruyama et al., 2007a). Mice were epicutaneously treated with 160 µl of an FITC solution (0.5% [w/v] in one of the solvents) on shaved forelimb skin. The solvents were acetone alone, DBP (50% [v/v] in acetone), menthol (10% [w/w] in acetone), carvacrol (5% [v/v] in acetone), AITC (5% [v/v] in acetone), CNA (20% [v/v] in acetone), and camphor (20% [w/w] in acetone). After 24 h, brachial lymph nodes were obtained and pooled for each condition. Single-cell suspensions of lymph nodes were prepared in DMEM by gentle teasing with needles. After washing in phosphate-buffered saline containing 0.1% BSA and 0.1% NaN₃, 2×10^6 cells were incubated with PE-conjugated anti-CD11c mAb (2 µg/ml) for 30 min at 4 °C. After washing, the cells were suspended in the same buffer. A total of 5×10^5 cells was examined with a flow cytometer (BD FACS

Cansto II; BD Biosciences, San Jose, CA) using gates for forward and side scatter to collect signals of cell-associated fluorescence. Isotype controls were used to monitor non-specific binding of antibodies. Data were analyzed with FACSDiva software. Each datum is representative of three independent experiments.

Statistics

To compare significant differences between multiple groups, one-way ANOVA and then Dunnett's test were employed. Curve fit and analysis were performed by using a Graphpad Prism (Graphpad Software, San Diego, CA).

Results

TRPA1 agonists induced calcium influx into TRPA1-expressing CHO cells

We previously demonstrated that DBP is an agonist as to TRPA1 (Shiba et al., 2009). Although menthol is known as a TRPM8 agonist (McKemy et al., 2002), it has also been shown to exhibit bimodal activation of TRPA1 (Karashima et al., 2007). Calcium influx into TRPA1-CHO cells but not into ones not expressing TRPA1 (T-Rex) was used as a measure of TRPA1 agonistic activity. TRPA1 agonists (AITC, carvacrol, CNA and DBP) significantly increased the intracellular calcium level of TRPA1-CHO cells in a dose-dependent manner (Fig. 1). The medial effective concentrations (EC_{50}) for TRPA1 activation by AITC, CNA and DBP were calculated to be 370 nM, 5.7 μ M and 1.6 μ M, respectively. Menthol exhibited TRPA1 agonist activity between 10 and 300 μ M. The results suggest that menthol exhibits TRPA1 agonistic activity in an appropriate concentration range, but that the activity decreases with higher concentrations. Non-specific calcium influx into T-Rex cells was seen for high concentrations of carvacrol and menthol. Because of their non-typical dose-response curves, EC_{50} was not determined for carvacrol or menthol. Camphor, reported to be a transient receptor potential vanilloid (TRPV) 3 agonist

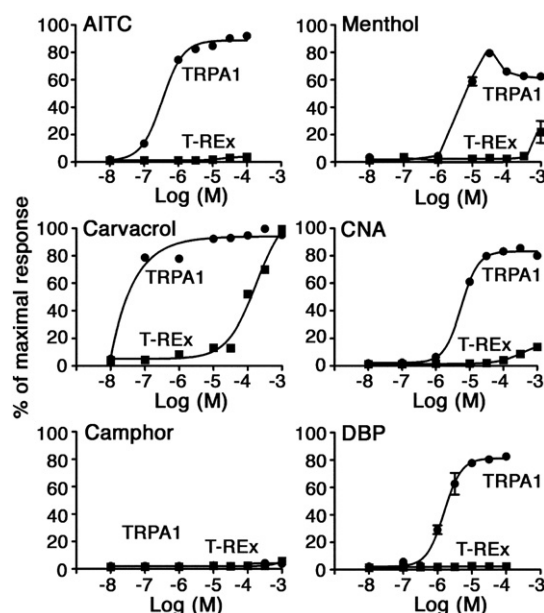


Fig. 1. Elevation of the intracellular calcium level in TRPA1-CHO cells caused by TRPA1 agonists. The concentrations (abscissa) of samples are plotted against the percentages of the maximal calcium responses (ordinate) in TRPA1-CHO cells or control T-Rex CHO cells. The samples were AITC, menthol, carvacrol, CNA, camphor and DBP. Each datum shows the mean $\pm$ SE ($n=4$).

and not to activate TRPA1 (Xu et al., 2005), did not induce any calcium response regardless of the expression of TRPA1.

Non-electrophilic TRPA1 agonists enhanced DC-trafficking

The sensitization phase of FITC-induced CHS is initiated by trafficking of FITC-presenting DCs. We examined the effects of non-electrophilic TRPA1 agonists because DBP may act as a non-electrophilic agonist. A solution of FITC in acetone containing DBP (50% [v/v]), menthol (10% [w/w]), or carvacrol (5% [v/v]) was painted on shaved forelimbs, and draining lymph node cells were analyzed by flow cytometry after staining with anti-CD11c. DBP (positive control), menthol and carvacrol significantly increased FITC⁺CD11c⁺ cell-trafficking as compared with under the control (acetone alone) conditions (Fig. 2).

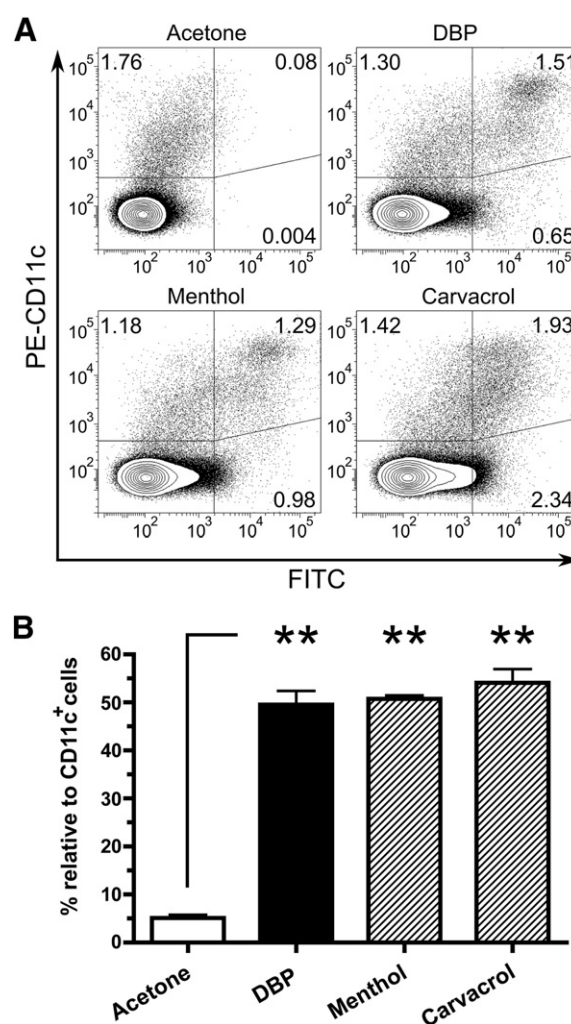


Fig. 2. Effects of non-electrophilic TRPA1 agonists on DC-trafficking. A. Representative cytograms showing DC-trafficking. Twenty-four hours after skin application of 0.5% FITC dissolved in acetone in the presence of 50% DBP, 10% menthol or 5% carvacrol, or in acetone alone, draining lymph node cells were analyzed for FITC and CD11c by flow cytometry. Dots in the plot represent the signals of individual cells. The numbers in the respective areas are the percentages of cells relative to total lymph node cells. B. Summary of DC trafficking. The percentages of FITC⁺CD11c⁺ cells relative to total CD11c⁺ cells (ordinate) were calculated from the numbers in the respective areas as shown in the panel A. For example under the DBP condition, 1.51% was FITC⁺CD11c⁺ and $1.51 + 1.30 = 2.81\%$ was CD11c⁺. Thus, $1.51/2.81 \times 100 = 53.7\%$ was positive for FITC among total CD11c⁺ population in this particular experiment. Each bar in the panel B represents mean $\pm$ SE in three independent experiments (** $P < 0.01$ vs. acetone).

Electrophilic TRPA1 agonists also enhanced DC-trafficking

AITC (5% [v/v]) and CNA (20% [v/v]) were tested as electrophilic TRPA1 agonists as to DC trafficking. As a control, camphor (20% [w/w]) was tested as a TRPV3 agonist exhibiting antagonistic activity toward TRPA1 (Xu et al., 2005), because menthol, carvacrol and CNA are known to activate TRPV3 as well (Xu et al., 2006). CNA significantly increased the trafficking of FITC⁺CD11c⁺ cells to draining lymph nodes compared with in the acetone control (Fig. 3). This increase was relatively less than those induced by non-electrophilic TRPA1 agonists (Fig. 2B). In the case of AITC, there was no increase in the trafficking of

FITC⁺CD11c⁺ cells. Camphor did not significantly increase the DC-trafficking (Fig. 3).

Enhancing effects of TRPA1 agonists on FITC-induced CHS

To examine the enhancing effects of TRPA1 agonists on sensitization, an ear-swelling test was performed. Mice were sensitized with an FITC solution in acetone containing a TRPA1 agonist (DBP, menthol, carvacrol or CNA) or camphor. One week after the last sensitization, FITC dissolved in acetone-DBP (1:1) was painted on an ear auricle, and then ear swelling was measured at 24, 48 and 72 h after the challenge. Acetone/DBP was used as the solvent during the elicitation phase. The lack of immunogenicity of DBP and the lack of an ear-swelling response due to the irritation caused by DBP have been demonstrated (Imai et al., 2006; Sato et al., 1998). At 24 h, the ear-swelling response was significantly high in mice that had been sensitized in the presence of a TRPA1 agonist as compared with in the absence of an agonist (Figs. 4A, B and C). Dose-response experiments revealed that a 10% (w/w) solution of menthol produced a higher response than a 50% (w/w) one, while 1% (w/w) was not effective (Fig. 4A). DBP (50% [v/v]), carvacrol (5% [v/v]), and CNA (20% [v/v]) also augmented the response. On the other hand, camphor (20% [w/w]) did not exhibit a significant effect. The ear swelling peaked at 24 h and then decreased with time (Fig. 4D, E and F), and the time course was the same for all groups.

TRPA1 antagonist HC-030031 inhibited DBP-induced calcium influx into TRPA1-CHO cells

HC-030031 is a TRPA1-selective antagonist (Bessac et al., 2009; Eid et al., 2008). TRPA1-CHO cells were activated with various concentrations of AITC, CNA or DBP in the presence of increasing concentrations of HC-030031. Higher doses of AITC or CNA were required to obtain the same level of calcium influx as the HC-030031 concentration increased (Figs. 5A and B). The results are interpreted as competitive antagonism. In the case of DBP, low concentrations of HC-030031 increased EC₅₀ for TRPA1 activation, while a high concentration (10 μ M) prevented the maximal level of calcium influx (Fig. 5C). This pattern of inhibition is interpreted as non-competitive or irreversible antagonism with a receptor reserve. The results demonstrated that HC-030031 is a potent antagonist for TRPA1 activation induced by DBP *in vitro*.

HC-030031 suppressed the enhancing effect of DBP on sensitization to FITC

We then examined whether or not pretreatment of mice with HC-030031 inhibited the enhancing effect of DBP on sensitization to FITC. We first re-examined the dose-response of DBP and found that 2% DBP consistently enhanced the efficiency of sensitization to FITC (ear-swelling response) as well as the trafficking of FITC-presenting DCs to draining lymph nodes (data not shown). The enhanced sensitization to FITC in the presence of 2% DBP (M-DBP group) was significantly inhibited by pretreatment with HC-030031 (HC-DBP group) 1 h before sensitization (Fig. 6). Inhibition was consistently observed at 24, 48 and 72 h after challenge. The enhancing effect of DBP under these experimental conditions was confirmed by the low ear-swelling response in the absence of DBP (M-A group) during sensitization (Fig. 6).

Discussion

During studies on phthalate esters that exhibit enhancing effects on the Th2-dominant FITC-induced CHS mouse model, we discovered that these phthalate esters are able to activate TRPA1 on neurons as well as on cells artificially expressing TRPA1. Here we found that several TRPA1 agonists, besides phthalate esters, enhance efficient skin

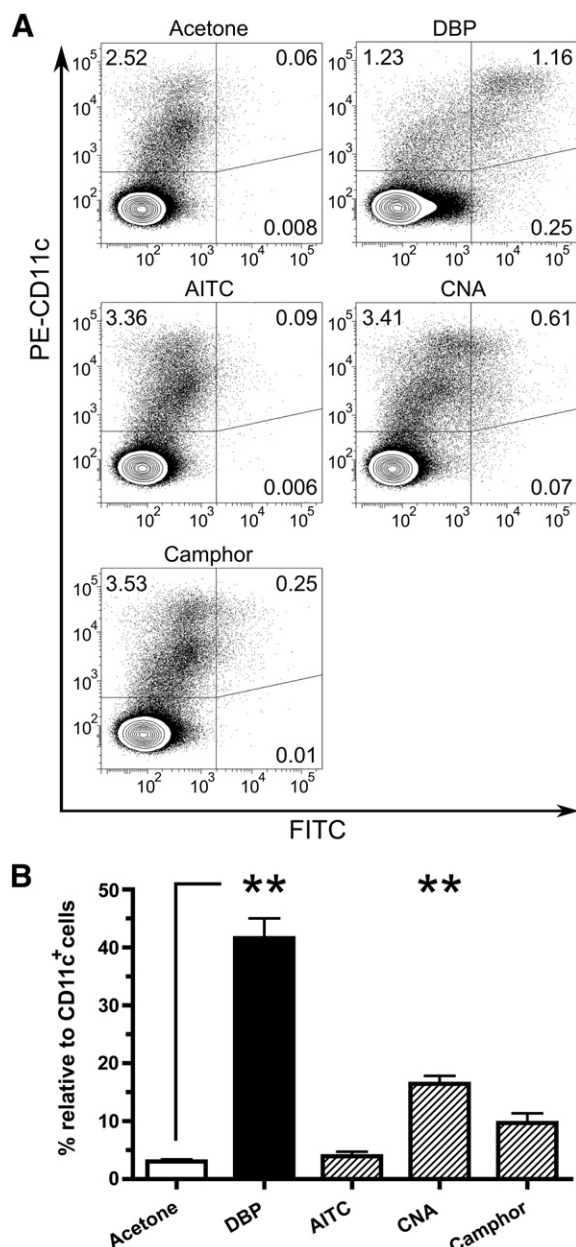


Fig. 3. Effects of electrophilic TRPA1 agonists and a TRPV3 agonist (camphor) on DC-trafficking. A. Representative cytograms showing DC trafficking. Twenty-four hours after skin application of 0.5% FITC dissolved in acetone in the presence of 50% DBP, 5% AITC, 20% CNA or 20% camphor, or in acetone alone, draining lymph node cells were analyzed for FITC and CD11c by flow cytometry. The numbers in the respective areas are the percentages of cells relative to total lymph node cells. B. Summary of DC trafficking. The percentages of FITC⁺CD11c⁺ cells relative to total CD11c⁺ cells (ordinate) were calculated from the data of panel A as described in the legend to Fig. 2. Each bar in the panel B represents mean $\pm$ SE in three independent experiments (** P < 0.01 vs. acetone).

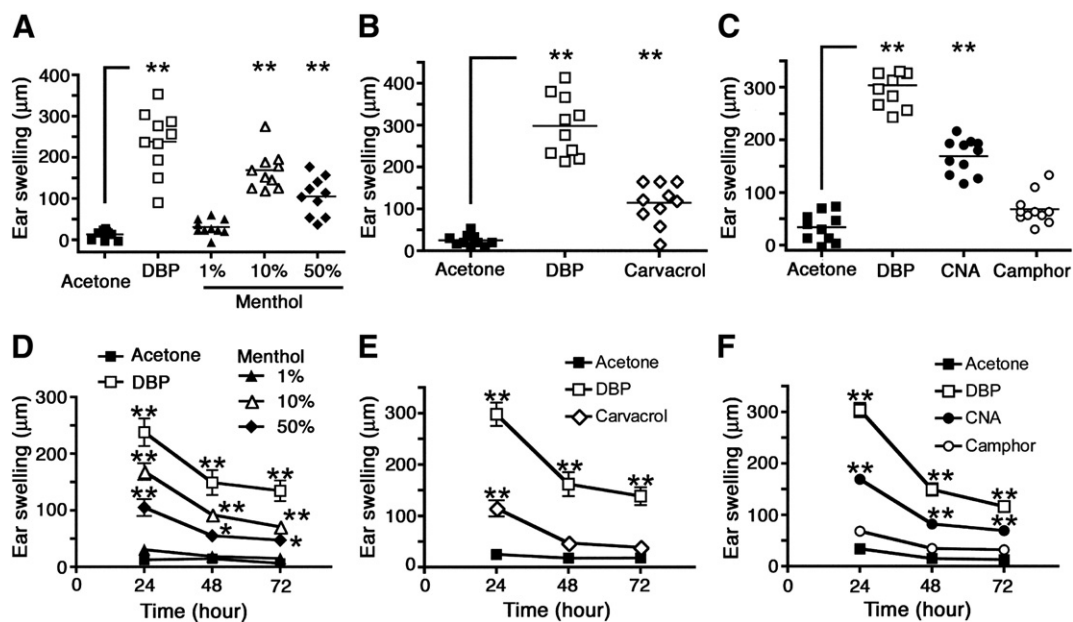


Fig. 4. Effects of TRPA1 agonists on skin sensitization to FITC. Mice were sensitized with 0.5% FITC dissolved in acetone in the presence of a TRPA1 (50% DBP; 1, 10 or 50% menthol; 5% carvacrol; or 20% CNA) or TRPV3 (20% camphor) agonist, or FITC in acetone alone (acetone) on days 0 and 7. On day 14, mice were challenged on an ear auricle with FITC dissolved in acetone/DBP. The ear-swelling response at 24 h after challenge in individual mice (each point) and means (horizontal bars) are shown (panels A, B and C). The time course of ear swelling is shown (panels D, E and F). Each symbol represents the mean $\pm$ SE ($n=9-11$). * $P<0.05$; ** $P<0.01$ compared with the acetone control.

sensitization to FITC in the CHS reaction and promote trafficking of FITC-presenting DCs to draining lymph nodes.

As non-electrophilic substances, menthol and carvacrol were shown to promote the trafficking of FITC-presenting DCs and to enhance the sensitization to FITC. Menthol has been shown to be a TRPM8 agonist (McKemy et al., 2002), but recent studies also revealed its activity as a TRPA1 agonist (Karashima et al., 2007). In this study, we also confirmed that menthol induced calcium influx into TRPA1-CHO cells (Fig. 1). The dose–response to menthol indicated that a higher concentration (50% [w/w]) was less effective than an intermediate one (10% [w/w]), as revealed by the ear-swelling response (Fig. 4). This is consistent with results showing the bimodal action of menthol on TRPA1 (Fig. 1) (Karashima et al., 2007).

The ear-swelling response differed among non-electrophilic agonists, whereas there was no difference in the percentage of FITC⁺CD11c⁺ cells among total CD11c⁺ cells in the draining lymph nodes (Fig. 2B). Although the percentages were the same, there was some difference in the DC subsets after FITC-sensitization in the presence of carvacrol or DBP. Skin DCs are heterogeneous as to phenotype and function, and some DC subsets exert a regulatory role in CHS. Although there is some controversy, Langerhans cells have been reported to exert a regulatory role in skin sensitization (Fukunaga et al., 2010; Kaplan et al., 2005). In addition, CD103-negative dermal DCs have been shown to produce retinoic acid constitutively and to be involved in the induction of Foxp3⁺ regulatory T cells (Guilliams et al., 2010). Carvacrol preferentially promoted the trafficking of a DC population with a moderate level of CD11c expression as compared with in the case of DBP (Fig. 2A). Although more detailed analyses are needed, this DC population may comprise DCs with poor antigen-presenting activity or with a regulatory role in the immune response.

AITC, a well-known TRPA1 agonist, did not promote FITC-presenting DC-trafficking (Fig. 3). It is not clear why the presence of AITC did not facilitate sensitization to FITC like that of other TRPA1 agonists did. Instead, topical pretreatment with AITC suppressed the enhancing effect of DBP on sensitization to FITC (Maruyama et al., 2007a). A higher

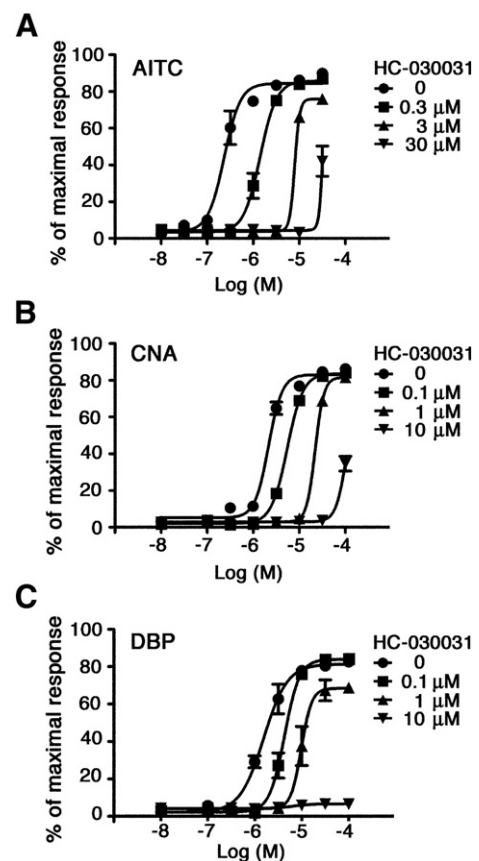


Fig. 5. TRPA1 antagonist HC-030031 inhibits DBP-induced calcium influx into TRPA1-CHO cells. TRPA1-CHO cells were stimulated with various concentrations of a TRPA1 agonist, AITC (panel A), CNA (panel B), or DBP (panel C), in the absence or presence of various concentrations of HC-030031 (shown in each panel). The concentrations of an agonist (abscissa) were plotted against the percentage of the maximal calcium response (ordinate). Each datum shows the mean $\pm$ SE ($n=4$).

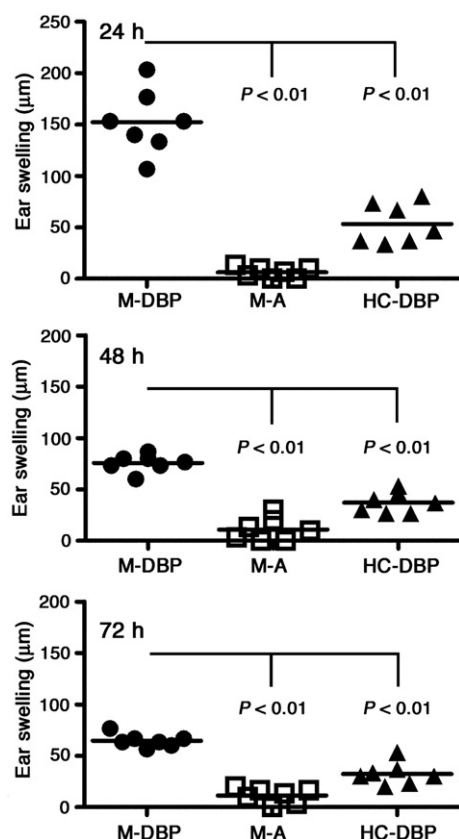


Fig. 6. TRPA1 antagonist HC-030031 inhibits the enhancing effect of DBP on skin sensitization to FITC. Mice were pretreated with 4 mg of HC-030031 or the vehicle, and then sensitized with 0.5% FITC dissolved in acetone or in 2% DBP-acetone on days 0 and 7. After challenge with 0.5% FITC on day 14, the ear-swelling responses of individual mice (each point) at 24, 48 and 72 h were examined. The experimental groups were as follows: M-DBP, vehicle (0.5% methylcellulose)-treated and sensitized with FITC in 2% DBP-acetone; M-A, vehicle-treated and sensitized with FITC in acetone; and HC-DBP, HC-030031-treated and sensitized with FITC in 2% DBP-acetone. The horizontal bars represent the mean values for individual mice ($n=7$). $P<0.01$ compared with the M-DBP group.

concentration of AITC may be needed, but the toxicity of AITC prevented us from testing concentrations higher than 5% (v/v). Because both AITC and FITC have an isothiocyanate group, another possibility is that AITC may compete with FITC in the formation of covalent bonds between proteins and FITC, and thereby inhibit the uptake of FITC-modified proteins by skin DCs. It is an interesting issue as to what attributes of AITC are involved in the desensitization of TRPA1 rather than in the enhancement of sensitization to FITC.

Menthol, carvacrol and CNA are also known to activate TRPV3 (Xu et al., 2006). There is a possibility that the enhancing effect on sensitization is mediated through TRPV3. To test this possibility, camphor, which is a TRPV3 agonist and a TRPA1 antagonist (Xu et al., 2005), was employed. In the present study, we found that camphor did not induce calcium influx into TRPA1-CHO cells (Fig. 1). Camphor also did not promote DC-trafficking (Fig. 3) or augment the ear-swelling response significantly (Fig. 4). These results suggest that the involvement of TRPV3 is minor, if any.

We previously demonstrated that phthalate esters also activate TRPV1 (Shiba et al., 2009). However, phthalate esters are more potent activators of TRPA1 than of TRPV1 (Shiba et al., 2009). Another group reported that TRPV1 activation might lead to an inhibitory effect during oxazolone-induced CHS (Bánvölgyi et al., 2005). In addition, camphor is also known to activate TRPV1 (Xu et al., 2005). Judging from these findings, TRPA1 is likely to play a more important role than TRPV1 during the sensitization to FITC.

Another unsolved problem was that even though DBP has been shown to activate TRPA1, the involvement of TRPA1 has not been directly demonstrated *in vivo*. In this study, we first demonstrated that the activation of TRPA1 by DBP was inhibited by HC-030031, as in the case of TRPA1 activation by other agonists (Fig. 5) (Bessac et al., 2009). Second, when mice were treated with HC-030031 before sensitization to FITC in the presence of DBP, the ear-swelling response was significantly suppressed (Fig. 6). These results constitute additional evidence of the TRPA1 involvement in the enhancing effect of phthalate esters on sensitization.

The downstream mechanisms after TRPA1 activation leading to enhanced DC-trafficking and FITC-induced CHS are not fully understood. One of the possibilities is that neuropeptides, such as calcitonin gene-related peptide (CGRP), are released from peripheral nerve endings following TRPA1 activation (Bautista et al., 2005). CGRP was reported to induce the release of TNF- α from mast cells (Niizeki et al., 1997). Mast cell-associated TNF was shown to promote DC migration (Suto et al., 2006). In our previous experiments, sensitization to FITC in the presence of DBP was suppressed by treatment with a CGRP antagonist at the site of skin sensitization (Maruyama et al., 2007a). A recent study suggested that CGRP suppresses 2,4,6-trinitrochlorobenzene (TNCB)-induced CHS but enhances FITC-induced CHS (Mikami et al., 2011). The immune response could be differently regulated by neuropeptides depending on the nature of the haptens or the nature of the immune responses. Thus, TNCB-induced CHS has been shown to be Th1-driven (Hayashi et al., 2001), but FITC-induced CHS to be Th2-driven (Dearman and Kimber, 2000). Neuropeptide release from sensory neurons may play a key role as to the connection between the nervous and immune systems.

Recently, two groups reported that DBP induced the production of thymic stromal lymphopoietin (TSLP) by keratinocytes (Larson et al., 2010; Shigeno et al., 2009). TSLP, which is produced by epithelial cells, is known as a cytokine associated with Th2-type responses (Ziegler, 2010). However, the mechanism of TSLP production upon exposure to DBP was not determined in those studies. On the other hand, recent studies revealed the expression of TRPA1 on human keratinocytes (Atayan et al., 2009). There is a possibility that TRPA1 activation on keratinocytes induces the production of TSLP. Thus, direct effects of TRPA1 agonists on keratinocytes followed by TSLP production could be another possible mechanism for the enhanced skin sensitization to FITC. It is an interesting issue as to whether or not TRPA1 agonists induce a calcium response in keratinocytes. It would also be interesting to examine whether or not TRPA1 agonists induce a calcium response in DCs themselves, because possible expression of some TRP channels has been reported (Basu and Srivastava, 2005). Further investigations are required to clarify these issues.

We focused on the role of TRPA1 in the enhancing effect on FITC-induced CHS. We do not rule out a possibility that skin allergen itself may have a capacity of TRPA1 activation that leads to an efficient sensitization. It should be noted that TRPA1 agonistic DBP and di-*n*-propyl phthalate did not facilitate skin sensitization to dinitrofluorobenzene or oxazolone. On the other hand, these phthalate esters enhanced skin sensitization to eosin 5-isothiocyanate, rhodamine B isothiocyanate and phenethyl isothiocyanate (Matsuda et al., 2010). The question of what attributes of haptens are required for the enhanced sensitization under the influence of TRPA1 pathway remains to be solved.

Chemicals with enhancing effects on sensitization to other allergens or haptens could be risk factors for allergies. It will be important to identify the targets of such chemicals. We propose TRPA1 as a candidate of such targets. Unidentified TRPA1 agonists may exist in the environment that facilitate the development of allergies in combination with immunogenic allergens or haptens. TRPA1 activation will become a useful marker for predicting such risks involved in allergic responses to some other chemicals.

Conflict of interest statement

The authors have no conflict to disclose.

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