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Topical effects of roflumilast on 1-chloro-2,4-dinitrobenzene-induced atopic dermatitis-like skin lesions in NC/Nga mice

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Phosphodiesterase-4 (PDE-4) inhibitors have a wide range of anti-inflammatory and immunomodulatory activities. Here we examined the effects of roflumilast, a well-known PDE-4 inhibitor, on 1-chloro-2,4-dinitrobenzene (DNCB)-induced atopic dermatitis-like lesions. Roflumilast inhibited DNCB-stimulated IL-1α secretion in HaCaT cells, and reduced ear thickness and lymph node weights in BALB/c mice sensitized with DNCB. Topical application of roflumilast to DNCB-induced atopic dermatitis-like skin lesions of NC/Nga mice ameliorated intensity scores and dorsal skin thickness, in parallel with reduced tissue IL-1β levels and epidermal hyperplasia. On the other hand, no effect on IgE and IL-4 was observed upon roflumilast treatment. Taken together, roflumilast showed beneficial effects against DNCB-induced atopic dermatitis.

1. Introduction

Atopic dermatitis (AD) is a non-contagious chronic inflammatory skin disease that commonly occurs in children and is characterized by symptoms such as itching, erythema, edema, oozing, crusting and lichenification. Despite intensive research on the pathogenesis of AD, the exact etiology and underlying mechanism of AD remain unclear. On the other hand, common characteristics such as increased mast cells and CD4+ T cells in association with IL-4 and IL-5 and increased IgE levels are observed in AD patients (Yamada et al. 1995; van Bever 1992; Leung 1992).

Phosphodiesterase-4 (PDE4) is an enzyme responsible for the hydrolysis of 3′,5′-cyclic adenosine monophosphate (cAMP); it is predominantly expressed in inflammatory cells (Dent et al. 1994; Gantner et al. 1997) and has been an attractive target for the treatment of respiratory diseases (Torphy 1998). Indeed, several PDE-4 inhibitors are currently in development as therapeutic agents for asthma and chronic obstructive pulmonary disease (COPD) (Spina 2008), and roflumilast (Daxas®), a representative PDE-4 inhibitor, is currently under review for clinical approval for the treatment of COPD (Cazzola et al. 2010). On the other hand, high PDE-4 activation has been observed in leukocytes of AD patients, and treatment with PDE4 inhibitors can lead to the improvement of allergic dermatitis under experimental conditions in BALB/c mice (Baumer et al. 2002; Harada et al. 2006).

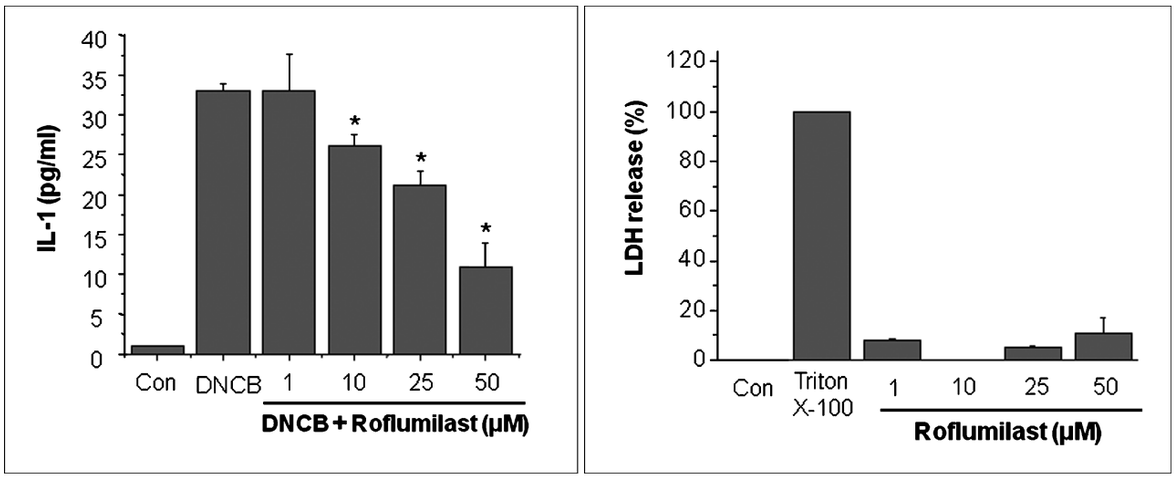
Many murine allergic dermatitis models have been established by repeated antigen exposure. The NC/Nga mouse is an inbred strain with a characteristic phenotype of spontaneous dermatitis; these mice develop skin lesions resembling AD in ambient laboratory conditions but not in specific-pathogen-free (SPF) conditions (Matsuda et al. 1997; Aioi et al. 2001; Suto et al. 1999). In this model, serum IgE and IL-6 levels are spontaneously increased under conventional conditions (Sudo et al. 2001). However, due to a low incidence and a longer development time of spontaneous dermatitis under conventional conditions, chemical antigens such as 2,4-dinitrofluorobenzene (DNFB) have been applied epicutaneously to induce a contact hypersensitivity reaction in NC/Nga mice (Tomimori et al. 2005).

In the present study, we investigated the topical effects of roflumilast, a representative PDE-4 inhibitor, on atopic dermatitis-like skin lesions in NC/Nga mice. 1-Chloro-2,4-dinitrobenzene (DNCB) was chosen as a sensitizing chemical for NC/Nga mice based on previous studies (Pokharel et al. 2008; Baumer et al. 2004), and AD parameters such as severity score, dorsal skin thickness, and IL-1β levels in skin tissues were assessed along with tissue histology. In separate experiments, the acute effects of roflumilast on DNCB-sensitized IL-1α secretion in HaCaT cells and on DNCB-stimulated increase in ear thickness and lymph node weight in BALB/c mice were examined. Based on the present study, topical application of roflumilast has protective effects against DNCB-induced atopic dermatitis-like skin lesions in NC/Nga mice, possibly by reducing the Th1-lymphocyte derived proinflammatory cytokine, IL-1.

2. Investigations and results

2.1. Effects of roflumilast on DNCB-stimulated IL-1α secretion in HaCaT cells

Before testing the *in vivo* efficacy of roflumilast against AD-like lesions, we preliminarily examined the effects of roflumilast on IL-1α production in HaCaT cells treated with DNCB. In HaCaT cells, DNCB (20 μM) induced IL-1α secretion of about 30-fold greater than that from the control group, whereas roflumilast reversed DNCB-induced IL-1α secretion



(A) (B)

Fig. 1: Downregulation of DNCB-induced IL-1α level by roflumilast in HaCaT cells (A) and cytotoxicity of roflumilast in HaCaT cells using LDH assay (B). To investigate the effect of roflumilast on DNCB-stimulated IL-1α secretion, 1×10^4 cells were exposed to 20 μM DNCB along with increasing concentrations of roflumilast for 12 hours. Culture media were removed to measure IL-1 α level. For cytotoxicity, 1×10^4 cells were plated into each well of 96-well plate and allowed to incubate with different concentrations of roflumilast. After 24-hour treatment, cytotoxicity was measured by LDH assay. $^*P < 0.05$ vs. DNCB alone

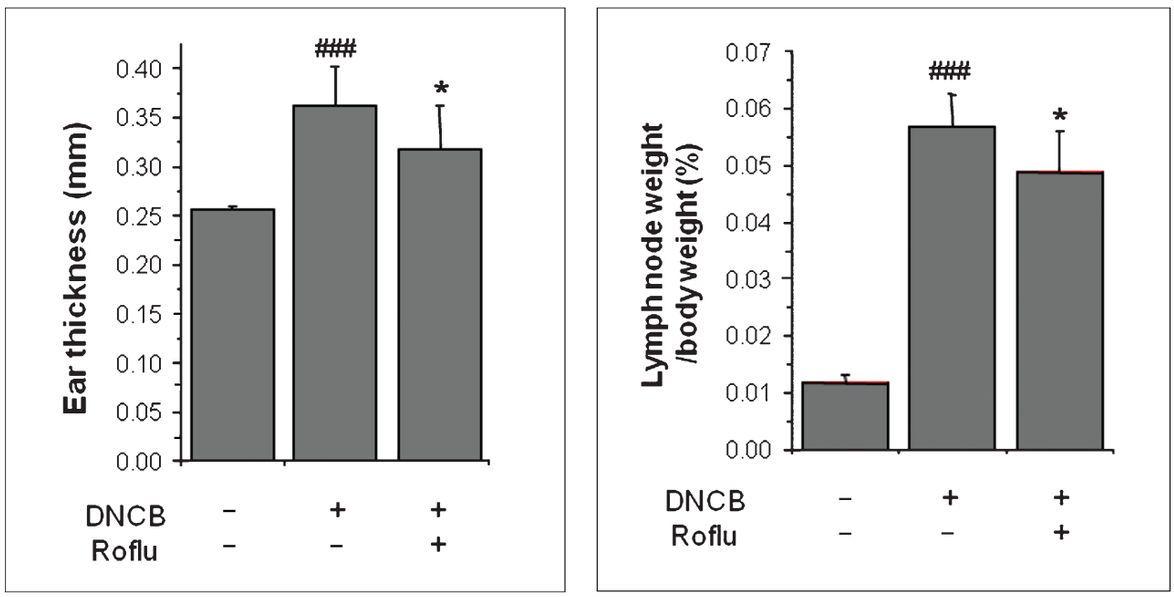
in a concentration-dependent manner with an approximate IC_{50} value of 25.3 ± 1.5 μM (Fig. 1A). DMSO (vehicle) itself did not increase the release of IL-1α, consistent with previous reports (Van Och et al. 2005; Vandebriel et al. 2005). No significant cytotoxicity was observed with up to 50 μM roflumilast (Fig. 1B), suggesting that roflumilast has an inhibitory effect on DNCB-induced IL-1α secretion.

2.2. Effects of roflumilast on DNCB-induced lymph node cell proliferation

The local lymph node assay was originally used to identify skin-sensitizing chemicals by measuring lymphocyte proliferation (Vandebriel et al. 2005). In the present study, we used this assay to determine the effect of roflumilast on DNCB-induced hypersensitivity reaction. After three successive DNCB treatments, ear thickness was increased by 0.1 mm, and lymph nodes were up to 6 times heavier than those in untreated mice. Topical application of roflumilast (4%) significantly reduced ear thickness (36% inhibition) and lymph node weight (19% inhibition) as shown in Figs. 2A and 2B.

2.3. Effects of roflumilast on DNCB-induced atopic dermatitis-like lesions in NC/Nga mice

Body weights of NC/Nga mice were monitored 2, 4, 6 and 7 weeks after sensitization with DNCB. No significant changes in body weight were observed between vehicle- and roflumilast-



(A) (B)

Fig. 2: Effect of roflumilast on DNCB-induced lymph node sensitization in BALB/c mice. The effect of roflumilast on ear thickness (A) and lymph node (LN) weight (B) was examined in BALB/c mice treated with DNCB. Mice were successively treated with DNCB and roflumilast (1 mg in 25 μl acetone) on days 1, 2 and 3. At three days after final treatment, LN weight and ear thickness were measured after excision of lymph node. $^*P < 0.05$ vs. DNCB alone, $^{\#\#\#}P < 0.001$ vs. control

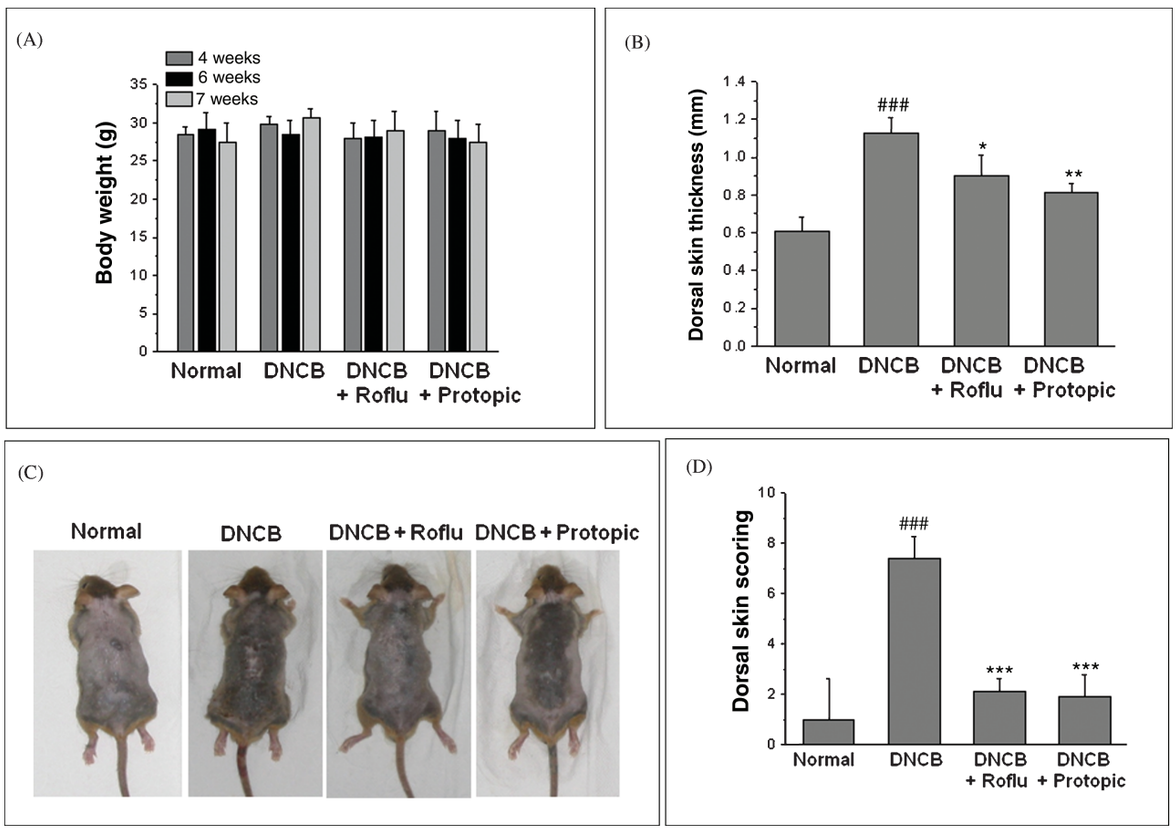


Fig. 3: Effects of roflumilast on body weight (A), dorsal skin thickness (B), macroscopic observation (C), and intensity score (D) in DNCB-induced NC/Nga mice. Body weights in each group of NC/Nga mice were monitored at week 2, 4, 6 and 7 after sensitization. At week 7 after sensitization, folded dorsal skin thickness was measured with a dial thickness gauge. Before sacrifice, the dorsal skin condition of NC/Nga mice was macroscopically observed and pictures of the skin were taken. Clinical skin severity scores were assessed at the end of the experiments based on the criteria described in Materials and Methods. $^{***}P < 0.001$ vs. DNCB alone, $^{\#\#\#}P < 0.001$ vs. control

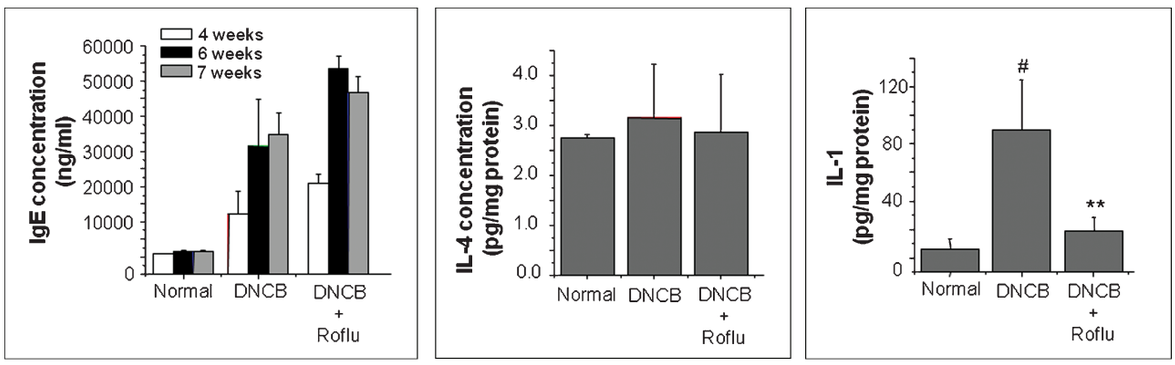
treated groups (Fig. 3A). Body weight increased normally with time during the test periods in all groups of NC/Nga mice, and no apparent adverse symptoms were observed at the doses used in either vehicle- or roflumilast-treated group.

At the end of the experiment (at week 7), skin severity score and dorsal skin thickness were assessed. DNCB application on the shaved skin increased skin thickness and caused outrageous skin appearance. Roflumilast treatment (0.4%) tended to decrease dorsal skin thickness although it was statistically insignificant (Fig. 3B).

Macroscopic observation of the dorsal skin of NC/Nga mice was conducted on the last day of the experiment. DNCB sensitization of the dorsal skin induced severe damage typical of AD-like skin lesions, but these lesions were considerably improved by roflumilast administration (Fig. 3C). Consistent with this, the skin severity score was significantly improved by roflumilast, almost completely recovering to normal level (Fig. 3D). Protopic® (0.1%), a commercially available AD agent and calcineurin inhibitor, exhibited effects comparable to roflumilast (results not shown).

2.4. Effects of roflumilast on IgE and cytokines in NC/Nga mice

To monitor serum IgE levels after challenge with DNCB, serum samples were collected at the indicated times from the mice



(A) (B) (C)

Fig. 4: Effects of roflumilast on serum IgE (A) and tissue IL-4 (B) and IL-1 (C) levels in DNCB-treated NC/Nga mice. Serum samples were collected at the indicated times, and total IgE levels were determined by ELISA Kit (Shibayagi, Shibukawa, Japan). IL-4 and IL-1β levels in dorsal skin tissues at week 7 after sensitization were determined by ELISA kit (R&D Systems, Wiesbaden, Germany). ** $P < 0.01$ vs. DNCB alone, $^{\#}P < 0.05$ vs. control

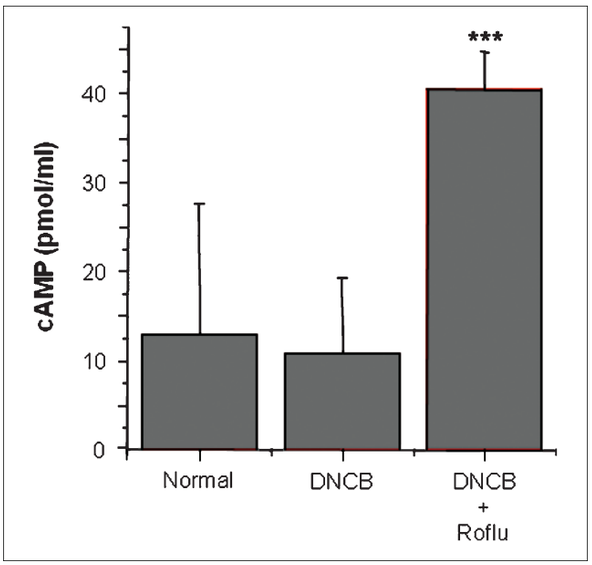


Fig. 5: Effects of roflumilast on serum cAMP levels in NC/Nga mice. Serum samples were obtained after sacrifice of NC/Nga mice at the end of the experiment. Total cAMP levels in NC/Nga mouse serum were determined by EIA kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). ***$P < 0.001$ vs. DNCB alone

following dermal application of either DNCB alone or DNCB with roflumilast. IgE levels were increased at weeks 6 and 7 upon DNCB treatment, but no reduction of IgE was observed with roflumilast treatment (Fig. 4A). IL-4 levels in dorsal skin tissues were not increased by DNCB with little alteration upon roflumilast treatment (Fig. 4B). In contrast, roflumilast significantly suppressed the tissue IL-1β level almost to that of the normal group (Fig. 4C).

2.5. Effects of roflumilast on blood cAMP levels

To determine whether roflumilast indeed increased cAMP levels, possibly via PDE-4 inhibition, blood cAMP levels following roflumilast treatment were measured by EIA. The cAMP concentration was significantly increased by about four-fold in the roflumilast-treated group (40 ± 5 pmol/ml) compared with that in the vehicle group (11 ± 8 pmol/ml) (Fig. 5), indicating that blood cAMP accumulated due to PDE4 inhibition with repeated applications of roflumilast.

2.6. Histological analysis

Consistent with macroscopic observation of dorsal skin tissues of roflumilast-treated mice, histological examination revealed that roflumilast ameliorated the increased thickness in epidermis and dermis and the loosened tissue context induced by DNCB (Fig. 6).

3. Discussion

AD is a hereditary and chronic skin disease generally characterized by skin inflammation, severe itching and dry skin. There might be a genetic predisposition for atopic conditions, but AD development is precipitated by environmental factors such as allergens, asthma or stress, and often develops into other allergic conditions (Helm 2004; Stevenson 2003). Although topical steroids are currently the mainstay for the treatment of AD, anti-inflammatory agents may be effective for AD therapy based on the general concept that AD is an inflammation-related skin disease.

PDE-4 is implicated in various inflammatory respiratory diseases such as asthma and chronic obstructive pulmonary disease, and PDE-4 inhibitors exhibit anti-inflammatory effects via cAMP accumulation in inflammatory cells (Doggrell 2006; Kwak et al. 2005). There have also been studies examining the effects of PDE-4 inhibitors on skin diseases. For instance, CP-80633 has been shown to be clinically effective as a topical treatment for AD (Hanifin et al. 1996), and some PDE-4 inhibitors including SB207499, RPR73401 and AWD 12-281 were effective in prevention and treatment of inflammatory reactions in a model of allergic dermatitis (Bauer et al. 2002; Ehinger et al. 2000). Recently, oral administration of PDE-4 inhibitors including roflumilast was shown to ameliorate 2,4,6-trinitro-1-chlorobenzene-induced dermatitis (Harada et al. 2008).

In this study, we aimed to expand the possible use of roflumilast in AD via locally delivery to circumvent the dose-limiting side effects of nausea and emesis following oral administration. First, we conducted a simple and rapid *in vitro* assay using HaCaT human keratinocytes to determine the effect of roflumilast on IL-1α secretion induced by DNCB. Keratinocytes have been shown to express PDE-4 and respond to allergen contact by producing proinflammatory cytokines, most notably IL-1α (Van Och et al. 2005; Vandebriel et al. 2005). When HaCaT cells were exposed to DNCB, the proinflammatory cytokine IL-1α was significantly released into media, whereas roflumilast treatment suppressed IL-1α secretion in a concentration-dependent manner with an approximate IC_{50} value of 25.3 ± 1.5 μM. In contrast to previous results (Van Och et al. 2005), DNCB (20 μM) itself did not induce a significant level of cytotoxicity as determined by LDH assay (results not shown).

Next, to verify the protective effect of roflumilast against DNCB-induced sensitization, we investigated the effect of roflumilast on DNCB-induced lymph node cell proliferation in the murine local lymph node assay (LLNA). Similar to a previous report (Kuricova et al. 2001), our results showed that DNCB application led to enlargement of local lymph nodes, indicating that hapten-specific lymphocytes in draining lymph nodes were activated and proliferated upon DNCB exposure. Topical application of roflumilast (4%) to mice diminished the DNCB-induced increases in auricular lymph node weight and ear thickness, suggesting that roflumilast may inhibit T cell proliferation stimulated by DNCB.

Based on these preliminary protective effects of roflumilast against DNCB sensitization *in vitro* and *in vivo*, we tested the efficacy of roflumilast on AD using an animal model. Over the last few decades, various animal models of AD have been developed; among these models, the skin lesions of inbred NC/Nga mice are clinically and histologically very similar to human AD-like lesions when the mice are maintained under conventional conditions but not under SPF conditions (Matsuda et al. 1997; Aioi et al. 2001). Furthermore, an epicutaneous application of chemical antigens such as 2,4-dinitrofluorobenzene (DNFB) evokes contact hypersensitivity reaction in NC/Nga mice, and prolonged dermatitis is observed even after cessation of DNFB application (Tomimori et al. 2005). While previous studies examining the efficacy of PDE-4 inhibitors in AD have usually used BALB/c mice treated with chemical sensitizers, we thought that NC/Nga mice would more closely mimic human atopic dermatitis based on previous determinations of skin barrier function and cutaneous ceramide contents (Aioi et al. 2001). Therefore, we adopted the protocol of Tomimori et al. (2005) with some modifications to investigate the efficacy of roflumilast in AD.

Sensitization and challenge with DNCB on dorsal skin of NC/Nga mice induced AD with a high severity score and elevated serum IgE levels. Topical application of 0.4% roflumilast, a dose similar to that used for other PDE-4 inhibitors (Baumer

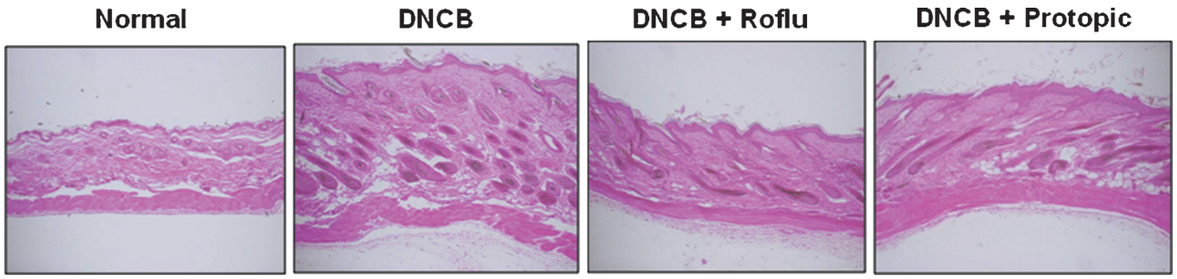


Fig. 6: Histological examination of DNCB-induced atopic dermatitis-like lesions in NC/Nga mice. At week 7, small pieces of dorsal skin were removed and stained with hematoxylin and eosin. H&E stained sections (×40) of dorsal skin of NC/Nga mice

et al. 2002) and protopic (0.1%), an immunosuppressant clinically used for AD treatment, improved both skin severity and thickness as compared with vehicle group. On the other hand, serum IgE, a hallmark of AD, was still elevated upon roflumilast treatment, and this finding is somewhat similar to previous reports showing that rolipram, another PDE-4 inhibitor, did not have inhibitory effects on serum IgE (Harada et al. 2006). Thus, it appears that the beneficial effects of roflumilast may be mediated by its actions on effector cells, rather than by direct action on IgE production. In particular, mast cells are important effector cells in anaphylaxis and IgE-associated allergic diseases such as atopic dermatitis, and sustained elevation of intracellular cAMP attenuated the release of inflammatory mediators from mast cells and basophils (Weston and Peachell 1998). As a confirmation of PDE-4 inhibition by roflumilast, serum cAMP level was highly increased by roflumilast.

The association of AD with increased PDE-4 levels is unclear, but it has been documented that PDE-4 activity in peripheral blood mononuclear leukocytes isolated from AD patients was significantly higher than that from age-matched non-atopic controls (Sawai et al. 1995; Butler et al. 1983; Cooper et al. 1985). An increase in PDE-4 activity by unidentified genetic aberrations leads to low levels of intracellular cAMP, which might cause basophils and mast cells to be hyperreactive with subsequent histamine release and leukotriene production. Conversely, administration of a selective PDE-4 inhibitor protected BALB/c mice from inflammatory damage in a model of allergic dermatitis (Baumer et al. 2002).

In the therapeutic strategies for AD, modulation of Th1/Th2 cytokine balance may be critical. In fact, skin biopsies from acute and chronic AD patients displayed differential cytokine expression (Hamid et al. 1994). Therefore, to address questions as to how roflumilast protects against skin damage from DNCB-induced inflammation, various cytokines were measured in skin tissues and serum. In the present results, Th1-lymphocyte-derived cytokine IL-1β was downregulated in the roflumilast-treated group, consistent with the *in vitro* inhibitory effects of roflumilast on IL-1α secretion in HaCaT cells. These findings are in accordance with the results reported by Baumer et al. (2002), which showed that SB207499 and AWD12-281, two known PDE-4 inhibitors, inhibited the increase of IL-1β. IL-1β concentration was increased in AD subjects and its elevation correlated with the severity of atopic dermatitis by inducing cell-mediated immune responses (Pellegrino et al. 1996), which reflects that IL-1β plays a key role in allergic inflammation. In addition, polymorphism of IL-1 was associated with atopy (Karjalainin et al. 2002), suggesting that IL-1 is specifically involved in allergic processes.

On the other hand, the IgE-promoting Th2 cytokine IL-4 was not affected by DNCB. A failure in IL-4 induction by repeated application of DNCB in the present study is in line with a previous report (Tomimori et al. 2005), and may be due to the fact that keratinocytes and Langerhans cells in skin tissue do not produce IL-4 (Morita et al. 2001; Shreedhar et al. 1998). We found that there was no difference in serum and skin tissue IL-4 levels between the normal and DNCB-induced groups, independent of IgE hyperproduction. IL-4 is known to be involved in class switch to IgE, but chronic AD skin lesions express significantly lower levels of IL-4 mRNA but higher levels of IL-5 mRNA than acute AD skin lesions (Hamid et al. 1994). In addition, IFN-γ produced by Th1 helper cells was not induced by DNCB in the present study (results not shown), in agreement with the observation that decreased IFN-γ in AD patients is related to increased IgE production. In contrast, the levels of IFN-γ were significantly higher following DNFB treatment (Tomimori et al. 2005), which might be due to differences in hapten application.

In summary, AD-like lesions in NC/Nga mice challenged with DNCB were improved by roflumilast, partly via modulation of the proinflammatory cytokine IL-1. Although some PDE-4 inhibitors have been shown to affect allergic dermatitis, this is the first report demonstrating the efficacy of topical application of roflumilast for atopic dermatitis symptoms in NC/Nga mice, an animal model closely resembling human AD. Further elucidation of the mechanism of action of roflumilast would be useful for broadening its therapeutic application for the treatment of AD.

4. Experimental

4.1. Chemicals

DNCB, *tert*-amyl alcohol and 2,2,2-tribromoethanol were purchased from Sigma (St. Louis, MO). Roflumilast was synthesized by the Korea Research Institute of Chemical Technology (Daejeon, Korea) with purity of >98%. For topical treatment, roflumilast was dissolved in acetone, and DNCB was dissolved in acetone and olive oil (AOO) (3:1, v/v). Both roflumilast and DNCB were dissolved in DMSO to examine cytotoxicity and IL-1α secretion in HaCaT cells. Avertin stock was prepared by dissolving 10 g of 2,2,2-tribromoethanol in 10 ml of *tert*-amyl alcohol, and a final working solution was prepared by dilution of 1 ml of stock solution with 39 ml of saline. Other chemicals were of analytical grade.

4.2. Animals

NC/Nga mice (male, 7 weeks of age) and BALB/c mice (female, 7 weeks of age) were obtained from Harlan Inc. (IN, USA) then bred and cared for in the barrier system in a temperature- and humidity-controlled room with a 12:12 hour light cycle. All animal experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH), and approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Chemical Technology.

4.3. In vitro effects of roflumilast on IL-1α secretion in HaCaT cells

HaCaT cells (Cell Lines Service, Eppelheim, Germany), a human keratinocyte cell line, were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin at 37 °C in a 5% CO_2 incubator. For IL-1α determination and cytotoxicity, HaCaT cells were plated at a density of 1×10^4/well into 96-well plates. One day after seeding, cells were preincubated with various concentrations of roflumilast for 1 h before 20 μM DNCB/DMSO treatment. Supernatants (50 μl) were taken after 12 h to measure total

IL-1α with a human IL-1α ELISA kit (R&D Systems, Wiesbaden, Germany). For cytotoxicity, lactate dehydrogenase (LDH) activity in culture media was measured after 48-hour roflumilast treatment by using a cytotoxicity detection kit (Roche, Indianapolis IN). Triton X was used as a positive control.

4.4. Local lymph node assay (LLNA)

Both ears of BALB/c mice (female, 7 weeks of age, n = 3/group) were treated with either 25 μl of 0.5% DNCB in AOO (3:1) or AOO alone. DNCB was consecutively applied once a day for three days in the presence or absence of roflumilast. Roflumilast (1 mg in 25 μl acetone) was topically painted onto both ears 1 h before DNCB treatment. Three days after final treatment, the lymph node was weighed, and the thickness of both ears was measured with a dial thickness gauge (Mitutoyo, Japan).

4.5. DNCB-induced atopic dermatitis

To establish an atopic dermatitis model, we adopted the protocol of Tomimori et al. (2005) except that we used DNCB instead of DNFB as a chemical sensitizer. NC/Nga mice (7 weeks old, male, n = 6-7/group) were anesthetized with avertin, and dorsal skin hair was shaved using a hair-clipper. Two days later, NC/Nga mice were sensitized by application of 300 μl of 1% DNCB in AOO (3:1) onto dorsal skin. Four days after sensitization, mice were challenged weekly for 5 weeks by application of 300 μl of 0.4% DNCB in AOO (3:1) at the same site and thereafter kept unchallenged for up to 7 weeks. Starting from week 3, 1 mg of roflumilast in 300 μl acetone was painted on the dorsal skin of DNCB-induced NC/Nga mice daily. To exclude any effects of vehicle itself, the DNCB control group was treated with acetone alone.

At week 7, NC/Nga mice were sacrificed by CO_2 asphyxiation to obtain serum and skin tissues, and clinical skin severity scores and dorsal skin thickness were assessed. Total scores were obtained by two independent observers in a blinded manner, and calculated as the average summation score of 5 symptoms (erythema, edema/population, oozing/crusting, excoriation and lichenification) in which each symptom was scored by the following criteria: no lesion, 0; mild, 1; moderate, 2; severe, 3. Skin thickness was determined by measuring folded dorsal skin with a dial micrometer.

The cAMP concentrations in serum were measured with the Biotrak cAMP enzymeimmunoassay (EIA) kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's protocol. Total serum IgEs were measured by the mouse IgE enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi, Japan). For the measurement of cytokines in skin tissues, dorsal skin was excised, cut into pieces, homogenized, and centrifuged at 12,000 rpm for 10 min. The resulting supernatants were assessed for IL-4 and IL-1β using mouse IL-4 and IL-1β ELISA kits, respectively (R&D Systems, Wiesbaden, Germany), and cytokine levels were normalized to the amount of total proteins as determined by Bradford assay. All ELISA kits were used according to the manufacturer's instructions.

4.6. Histology of dorsal skin

Small pieces of skin tissues were cut from the dorsal skin of mice on week 7 after sensitization, fixed in 4% formalin overnight, embedded in paraffin, sectioned by 8 μm and stained with hematoxylin and eosin. Images were captured at a magnification of 40x.

4.7. Statistical analysis

Values are expressed as the mean ± SEM of three experiments each carried out in triplicate. Data were statistically evaluated by using Student's *t*-test, in which *P* values less than 0.05 were considered significant.

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