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Original Article

Effect of topical application and intraperitoneal injection of oregonin on atopic dermatitis in NC/Nga mice

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Abstract: The diarylheptanoid, oregonin (ORE), which was isolated from the bark of *Alnus japonica* Steudel that grows natively in Korea, has been known to exert antioxidative, anti-inflammatory, anti-cancer and immune response inhibitory effects. The antioxidative effect of ORE was observed on the superoxide and 1,1-diphenyl-2-picrylhydrazyl radical, as well as on the expression of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-treated RAW264.7 macrophages. The statistically significant inhibitory action of ORE against production of cytokines induced by bacterial products or by interleukin (IL)-1β, free radicals and nitrogen species, and a corresponding increase in cellular calcium concentration because of ORE were confirmed in bone marrow and spleen dendritic cells that are known to play important functions in the development and advancement of atopic dermatitis (AD). It was thus expected that ORE would exert a beneficial effect in the treatment of AD. A study on the pharmaceutical benefits of ORE against AD has not yet been conducted *in vivo*. We therefore used an *in vivo* AD animal model, namely the NC/Nga mice, and by applying ORE onto the animals through skin application as well as intraperitoneal injection, we attempted to evaluate the benefits of ORE in this system. Evaluation of ORE was conducted by following the SCORE method to score the effect, as well as by measuring the Th2 cytokines IL-4, IL-5 and IL-13 levels from serum and lymphocytes, and IgE and eosinophil levels from serum. Additionally, the expression of mRNA and protein levels was estimated using real-time polymerase chain reaction and Western blotting analysis. The following categories of clinical evaluation, Th2 cytokines IL-4, IL-5 and IL-13 values, serum IgE levels, serum eosinophil levels, and mRNA and protein expression levels of iNOS and COX-2, were evaluated from topical application and intraperitoneal injection groups of ORE. The effects of ORE on AD in NC/Nga mice were confirmed as being similar to the positive control group, while a significant difference with the negative control group was observed. The results presented in this report suggest that ORE might be beneficial in the treatment of AD.

Key words: *Alnus japonica* – atopic dermatitis – diarylheptanoid – NC/Nga mice – oregonin

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Introduction

Diarylheptanoids are characteristic components of the genus Alnus (1) that have been used in oriental traditional medicine as remedies for fever, haemorrhage, diarrhoea and alcoholism (2). The diarylheptanoids show several biological activities including anti-oxidative (3,4), inhibitory activities of nitric oxide (NO) synthase (5) and cyclooxygenase-2 (COX-2) expression (6) as well as anti-inflammatory effects (7–9).

Atopic dermatitis (AD) is an immune disorder that occurs because of the involvement of genetic and environmental factors, and is also a chronic disease that is classified as intractable (10,11). AD is characterized by the increased eosinophil counts and the increased concentration of serum IgE, which is the representative indicator for AD (12,13). In the adaptive immunologic processes, helper T cells (Th cell) play a crucial role. Activated Th cells are divided into Th1- and Th2-subtypes according to the types and immune func-

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tions of cytokines. When the balance between these cells is broken, disease conditions are induced. For example, when the balance of Th1/Th2 is slanted to Th2, this leads to development of acute AD. When the Th2 cell is activated, the levels of so-called 'Th2 cytokine' – interleukin (IL)-4, IL-5 and IL-13 – increase. Among them especially IL-5 induces the synthesis of IgE from B cells, which contributes to the prolonged allergic reactions observed in AD.

Th2-related cytokines such as IL-4, IL-5 and IL-13 were increased and Th1-related cytokines such as IL-2 and interferon-gamma were reciprocally decreased (14–16).

Up to the present, AD has been regarded as an intractable dermatologic disorder, whose treatment modalities mainly include corticosteroid, anti-histamines and immune suppressants. Because of the side-effects developed during the therapy, and recurrence after cessation of the therapy, they had limited value as therapeutic agents. It is imperative that alternative and compensatory agents be developed promptly to treat this condition. Meanwhile, natural substance-based therapeutics were tried as potential therapeutic agents for AD (17–20).

NC/Nga mice show very similar traits noted in AD, including increased IgE level, chronic dryness and severe pruritis (21–23). They are generally adopted as animal experimental models for AD. Frequently, AD-like skin lesion, however, is not induced easily in NC/Nga mice under specific pathogen-free conditions. Thus, sensitization reaction via contact with an antigen like diphenycyclopropenone (DPCP) is often required (24).

In the current study, to develop a therapeutic agent for AD, oregonin (ORE; Fig. 1), which was diarylheptanoid glycoside isolated from the cortex of *Alnus japonica* (25) and known to exert several anti-inflammatory activities (5–7) and anti-atopic properties *in vitro*, which were inhibitory effect on the production of cytokine, the formation of reactive oxygen species and nitric oxide, and the change in intracellular Ca^{2+} levels in dendritic cells of bone marrow and spleen exposed to microbial products and IL-1β (26), was administered via topical application and intraperitoneal injection in NC/Nga mice.

Materials and methods

Materials

Animals

Female 5-week-old NC/Nga mice were purchased from Charles River Japan (Yokohama, Japan) and were maintained under conventional conditions. All animals were maintained on a 12-h light/12-h dark cycle with food and water freely available. The temperature of the colony room was maintained at 22–23°C and humidity 55 ± 15% (27–29). The animal care, handling and experimental procedures were carried out in accordance with a protocol approved by the Animal Care and Use Committee of the Chung Ang University of Korea.

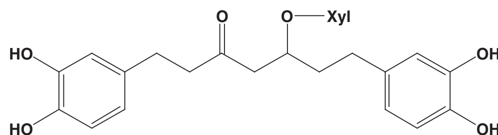

Figure 1. Chemical structure of oregonin.

Oregonin

Oregonin was isolated from the fresh bark of *Alnus japonica* (25) and was administered via topical application and intraperitoneal injection in NC/Nga mice.

Diphenylcyclopropenone

Diphenylcyclopropenone is well known as a contact allergen. It was used into in order to induce atopic-like skin lesions.

Methods

DPCP stimulation

The dorsal hair of the mice was removed and then stimulated for 10 weeks with DPCP under conventional conditions. NC/Nga mice were sensitized with DPCP (0.1%) on the back, once a week as previously described (24,30,31).

Clinical observation of AD

Clinical observation on the back of NC/Nga mice was measured for 4 weeks. The challenged parts of the mice was observed through the photograph. To compare the skin lesions of the mice, the clinical severity of the dermatitis was scored using previously described macroscopic diagnostic criteria, which are normally used for human AD. In brief, the severity of dermatitis was evaluated once a week. The development of (1) erythema/haemorrhage, (2) scarring/dryness, (3) oedema, (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate) and 3 (severe), respectively. The sum of the individual scores was taken as the dermatitis score (20,32).

Oregonin treatment

Phosphate Buffered Saline (PBS), 0.1% ORE, 1% ORE liquid solutions were intraperitoneally injected, twice a week and base cream and 1% ORE topical cream were applied to exposed skin on the back of the mice for an indicated day for 4 weeks.

Measurement of eosinophil count

Blood samples were collected at 20 weeks of age. Whole blood cell counts were conducted on 30 μl of capillary blood diluted sixfold with 150-μl saline. Microscopic differential counts were performed using Wright-Giemsa stained blood smears. Eosinophil counts were calculated from differential counts.

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Measurement of serum IgE
The amount of total IgE in the serum was determined by a sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) method as previously described (33–35). In brief, 96-well plates were coated with monoclonal anti-mouse IgE incubated overnight at 4°C, and then further treated with 2% (w/v) bovine serum albumin (BSA) dissolved in PBS containing 0.05% Tween 20 (PBS-T) for 2 h at room temperature to block any non-specific binding. Next, serial dilutions of the serum samples were incubated in the wells, in duplicate, for 1 h at room temperature. After rinsing three times with PBS-T, biotin-conjugated rat anti-mouse IgE Ab (LO-ME-2) was added to the wells, followed by the addition of streptavidin-peroxidase (Seikagaku Corp., Tokyo, Japan). After rinsing three times, the plates were developed using a substrate solution containing 0.04% *O*-phenylenediamine dissolved in phosphate citrate buffer (pH 5.0). The reactions were terminated by the addition of sulphuric acid. The absorbance was read using a microplate reader at 490 nm, and the amount of the Ig isotype was calculated by comparing it with the mouse IgE standard (Pharmingen, San Diego, CA, USA) (35,36).

Lymphocyte culture
Lymphocytes were isolated from spleen tissue. Red blood cells (RBC) were lysed using RBC lysis buffer (Sigma, St. Louse, MO, USA). The lysate was centrifuged at 12 000 rpm for 15 min at 4°C. The supernatant was removed and the cells were seeded at 1×10^6/well, which were then cultured in 24-well plates. The supernatant containing cultured lymphocytes was harvested and measured for the presence of cytokines.

Cytokine measurement
Blood samples were collected at 20 weeks of age. The supernatants of cultured lymphocytes were analysed for cytokine levels. In this measurement, IL-4, IL-5 and IL-13 were detected using a mouse cytokine enzyme immunoassay kit (R&D, Minneapolis, MN, USA) (37).

Tissue sampling
Tissues from the AD-like skin lesions were taken for measuring iNOS and COX-2 expression.

Real-time polymerase chain reaction
Preparation of primer. We synthesized the polymerase chain reaction (PCR) primers (Genotech, Daejeon, YG, Korea) using data in the Gene Bank. Primers were chemically synthesized using a DNA synthesizer (Pharmacia, Björkgatan, Uppsala, Sweden). Their sequences were as follows:

iNOS (65 bp)
5′-CTG ATG CCT CTT CCA GGT GT-3′ (sense)
5′-GAG GGA GCC CTT TCT GAA TC-3′ (anti-sense)

COX-2 (80 bp)
5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ (sense)
5′-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3′ (anti-sense)

GAPDH (93 bp)
5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ (sense)
5′-CCC TGT TGC TGT AGC CGT AT-3′ (anti-sense).

Total RNA was isolated from tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Basically, the procedure involved the addition of 1 ml of TRIzol reagent to the cells grown in a culture dish. After 5 min at room temperature, 0.2 ml of chloroform per ml of TRIzol reagent was added and the tubes were shaken vigorously by hand for 15 s. These were then incubated at 15–30°C for 3 min. The mixture was centrifuged at 12 000 rpm for 15 min at 4°C, and then the upper aqueous phase was transferred to a fresh tube into which the same amount of 2-propanol was added. After incubation at 4°C for 15 min, it was centrifuged with 12 000 rpm at 15 min (4°C). The supernatant was removed, washed with 500 μl of 70% ethanol and centrifuged at 12 000 rpm (4°C) for 5 min. The resulting RNA pellet was briefly dried. Purified RNA was dissolved in diethyl pyrocarbonate-distilled water. Total cellular RNA was reverse transcribed at 42°C for 30 min using a reverse transcriptase (TaKaRa, Otsu, Shinga, Japan), 10× buffer, 10 mM dNTP (dNTP mix), oligo dT primer, RNase inhibitor and 25 mM $MgCl_2$.

Two microlitres of each cDNA sample from the RT-PCR reaction was amplified by PCR in 25 μl of total volume containing 10× buffer 2.5 μl, 25 mM $MgCl_2$ 2.5 μl and 10 pmol (0.75 μl) primer. PCR was conducted with 10× Buffer for Taq polymerase (100 mM Tris–Cl pH 8.5, 400 mM KCl), 1 mM dNTP each, 50 mM $MgCl_2$, upstream primer (5 μM), downstream primer (5 μM), DNA template (less than 200 ng) and SyBr Green I.

Quantitative analysis
A densitometer was used for Western blotting of the protein products of *iNOS*, *COX-2* and *Actin* on DIG chemiluminescent film (volume of *iNOS*, *COX-2*/volume of *Actin* X100).

Western blotting
Tissue was lysed in a buffer containing 50 mM Tris–Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethanesulphonyl fluoride, 1 μg/ml aprotinin, 1% Triton ×100, centrifuged at 12 000 rpm (4°C) for 30 min. The supernatant was transferred into a new tube, and 30 μg of soluble protein was loaded in a 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis system with sample buffer containing 1 M Tris, glycerol 50%. The samples were heated at 95°C for 5 min prior to gel loading.

For iNOS and COX-2 detection, the electrophoretically resolved proteins were transferred onto nitrocellulose mem-

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branes (Osmonics, Milwaukee, WI, USA) at 0.16 A for 1 h. The membranes were washed three times with Tris-buffered saline Tween 20 (TBS-T), and blocked with 5% skim milk for 1 h at room temperature.

Following this, the membranes were incubated overnight at 4°C with goat anti-human iNOS and COX-2 polyclonal antibody (1:1000 in 5% BSA; Chemicon, Temecula, CA, USA) and then washed three times with TBS-T. The membranes were then incubated in secondary mouse anti-rabbit peroxidase conjugated antibody (1:2000 in blocking solution; Chemicon) for 1 h at room temperature.

Statistical analysis

All data are expressed as the mean ± SE. Statistical difference between the groups were determined by a factorial analysis of variance (ANOVA) followed by an unpaired t-test.

Results and discussion

Atopic dermatitis is a chronic inflammatory skin disease characterized by the hypersensitivity of the skin in response to various environmental and genetic factors (38). AD is developed mainly in infants and children, although its incidence has recently increased in adult patients. Major symptoms of this condition include severe pruritis, dryness and eczema. To date, because of a lack of the exact pathogenesis or definite treatment, studies associated with AD have been actively conducted worldwide. The currently known steroids, anti-histamines and immunosuppressants have disclosed various limitations in the efficacy of treatment and as such it is imperative that alternative and compensatory agents be developed promptly to treat this condition. In an attempt to develop an appropriate treatment agent for AD using a natural substance, we performed an *in vivo* experiment using NC/Nga mice to assess the anti-AD effect of ORE, which was already known to have various biological activities including antioxidation (3,4), inhibition of NO synthesis (5) and inhibition of COX-2 synthesis (6).

Oregonin, contained abundantly in the barks of *Alnus japonica,* was used for the current study in an attempt to make an objective evaluation of its anti-AD efficacy. A well-known animal experimental model with NC/Nga mice was used (21–23). Immune hypersensitivity was provoked using the contact as allergen, DPCP (24). Two methods of administering ORE were used, namely topical skin application and intraperitoneal injection.

Clinical evaluation

For the objective assessment of AD, the gross examination of skin lesions was performed using the SCORE method (28). ORE treatment significantly reduced the severity of AD-like lesions induced in NC/Nga mice. The severity of dermatitis was evaluated once a week. The development of (1) erythema/haemorrhage, (2) scarring/dryness, (3) edema, (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate) and 3 (severe). The sum of the individual scores was taken as the dermatitis score (Figs 2 and 3) (39). Clinical signs and symptoms seen in conventional NC/Nga mice began with itching, erythema and haemorrhage, followed by edema, superficial erosion, deep excoriation, scaling and dryness of the skin, and retarded growth. Total severity scores increased with ageing, and reached a clinical score of more than 13 out of 15 points in 20-week-old females.

Eosinophil level

Eosinophils, which play a crucial role in aggravating AD symptoms, infiltrated into the dermis within 2–6 h after contact with allergens. Approximately 24–48 h later, eosinophils secrete the eosinophil cationic protein and thereby inducing infiltration of other immune cells into the skin lesions (12,40), thus aggravating the symptoms of AD. Eosinophil levels were decreased after treatment of the mice with ORE. This indicated that ORE might be associated with improvement of eosinophil-related allergic diseases (Fig. 4).

IgE level

One of the characteristics often seen in the serum of patients with AD is an increased level of IgE. In recent years, this has been postulated as the key factor for pathogenesis of AD and the possibility of using this as a prognostic indicator has also been proposed (13,41). The increased level of IgE is also a diagnostic indicator for AD. As shown in Fig. 4, ORE treatment, both topically and intraperitoneally, significantly down-regulated the IgE levels in the NC/Nga mice.

Cytokine level

It was recently shown that the aetiology of AD was based on the abnormality of cytokines (42,43). The imbalance between Th1 and Th2 levels, i.e., the predominance of Th2

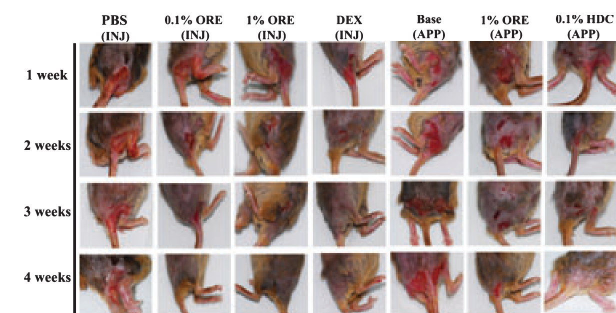

Figure 2. Clinical observation of AD-like skin lesion induced in NC/Nga mice during experimental periods. ORE treatment significantly reduced the severity of AD-like lesions induced in NC/Nga mice. Base, baseline; HDC, hydrocortisone cream; INJ, injection; APP, application. .

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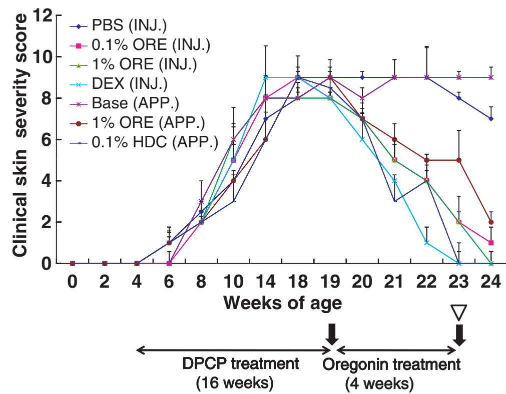

Figure 3. Changes of the total clinical severity score by topical application and intraperitoneal injection of ORE. Application of 0.1% DPCP on the back of the animals once at 1-week intervals induced the AD-like skin lesions. The total clinical severity score was defined as the sum of scores graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe) for each of the seven aspects; erythema, haemorrhage, oedema, excoriation, erosion, scaling and dryness. The results are expressed as means ± SD ($n = 5$). Black arrow denotes (↓) are blood sampling, Arrow denotes (▽) are tissue sampling. ORE, oregonin; DEX, dexamethasone; base, baseline; HDC, hydrocortisone cream; INJ, injection; APP, application. [Correction added after online publication 22 October 2009: Figure 3 graph was replaced.]

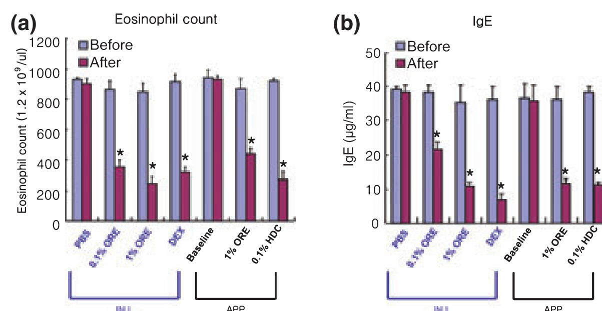

Figure 4. Effect of topical and intraperitoneal injection of ORE on eosinophil count and IgE. (**a**) Eosinophil levels were decreased after treatment of the animals with ORE. ORE may thus be associated with improvement of eosinophil-related allergic diseases. (**b**) ORE treatment, topically and intraperitoneally, significantly down-regulated the IgE levels in NC/Nga mice. The results are expressed as mean values ± SD (*$P < 0.01$, $n = 5$). ORE, oregonin; DEX, dexamethasone; base, baseline; HDC, hydrocortisone cream; INJ, injection; APP, application.

response, contributes to the development of acute AD skin lesions. The causes of imbalance between Th1 and Th2 have not yet been elucidated. For effective treatment of AD, Th2-related cytokines must be suppressed (14–16). Therefore, the rate of suppression of such Th2-related cytokines such as IL-4, IL-5 and IL-13 was measured in serum and supernatants of lymphocytes. In the ORE-treated group, the expression of cytokines was significantly inhibited. These results suggest that ORE might exert an immunoregulatory function associated with AD (Fig. 5).

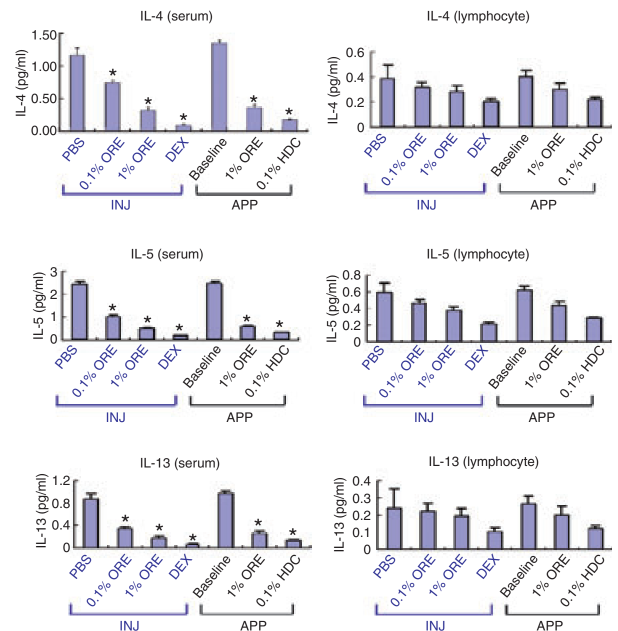

Figure 5. Effects of topical application and intraperitoneal injection of ORE on serum Th2 cytokine levels. In the ORE-treated group, the expression of cytokines was significantly inhibited. These results suggest that ORE might play an immunoregulatory role associated with AD. The results are expressed as mean values ± SD (*$P < 0.01$, $n = 5$). ORE, oregonin; DEX, dexamethasone; base, baseline; HDC, hydrocortisone cream; INJ, injection; APP, application.

iNOS and COX-2

Nitric oxide plays a role in vasodilation, neurotransmission, blood coagulation and immune regulation, and is mainly divided into cNOS and iNOS. Of these, iNOS is involved in the massive production of NO when it is activated by various cytokines or bacterial Lipopolysaccaride (LPS). An excessive presence of NO aggravates the inflammatory response. Therefore, one of the methods of examining the anti-inflammatory activity of a compound is to measure its effect on the rate of suppression of the synthesis of iNOS (44). COX is divided into COX-1 and COX-2. COX-1 plays a role in maintaining homeostasis within the body. In contrast, COX-2 is markedly expressed in inflammation-related cells in response to stimulations with cytokines in inflammatory or other immune reactions. Thus, the rate of suppression of the synthesis of COX-2 is also a well-known and universal method of testing the anti-inflammatory effect of various compounds (33).

The mRNA and protein expression levels of iNOS and COX-2 were inhibited in the ORE-treated group (Fig. 6). These results suggested that ORE might be effective in exerting anti-inflammatory activity in NC/Nga mice.

Conclusion

To develop an appropriate therapeutic agent for AD, ORE, which was isolated from the cortex of *Alnus japonica* and

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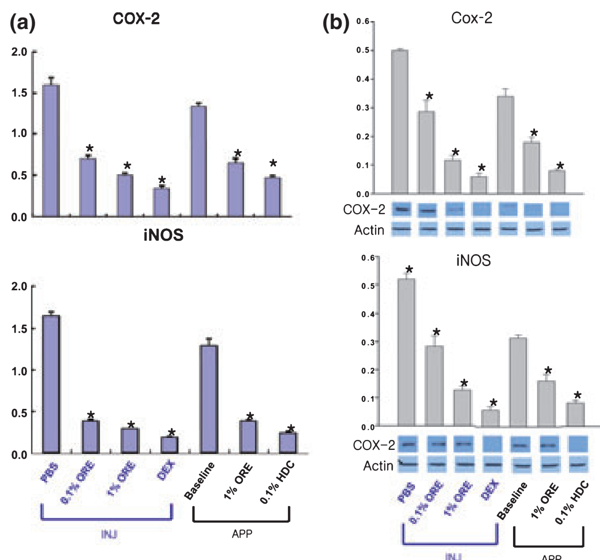

Figure 6. Effects of topical application and intraperitoneal injection of ORE on mRNA and protein expression of iNOS and COX-2. (a) These were determined via semi-quantitative RT-PCR. The mRNA expression levels of *iNOS* and *COX-2* were inhibited in the ORE-treated group. (b) These were determined via Western blotting. The protein expression levels of iNOS and COX-2 were inhibited in the ORE-treated group. These results suggested that ORE might be effective in exerting anti-inflammatory activity in NC/Nga mice. The results are expressed as mean values ± SD (*$P < 0.01$, $n = 5$). ORE, oregonin; DEX, dexamethasone; base, baseline; HDC, hydrocortisone cream; INJ, injection; APP, application.

known to have anti-atopic properties *in vitro*, was administered via a topical application and an intraperitoneal injection in NC/Nga mice. The clinical skin severity score and the indices of clinical findings were decreased after treatment of the animals with ORE. Eosinophil and IgE levels in the blood were also decreased after treatment with ORE. ORE also significantly reduced Th2 cytokine levels in the serum. In addition, the transcription and translation levels of both, the expression of COX-2 and iNOS in AD lesional skin, were significantly suppressed by ORE treatment ($P < 0.05$). These results demonstrate that ORE might be beneficial in the treatment of skin allergies such as AD.

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