INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by erythema, eruption, pruritus and hyperkeratosis accompanied with increased transepidermal water loss (TEWL) (Tsujii et al., 2009). AD has a complex etiology that involves abnormal immunological and inflammatory pathways that yield defective skin barrier, exposure to environmental agents, and neuropsychological factors (Jin et al., 2009). Epidermal barrier disruption increases the invasion of allergens and bacteria and worsens the inflammatory response. The epidermal barrier is developed when keratinocytes differentiate and secrete lamellar bodies. Protease activated receptor 2 (PAR2) belongs to a subfamily of G-protein coupled receptors (Bohm et al., 1996) and is activated through proteolytic cleavage by specific serine proteases, such as mast cell tryptase and trypsin. These serine proteases cleave the extracellular N-terminal peptide in humans. Subsequently, the cleaved peptide sequence, as a tethered ligand, activates the receptor, itself (Vergnolle et al., 1999). In skin, PAR2 is expressed in suprabasal layers of the epidermis (Nystedt et al., 1996). PAR2 expression is associated with the regulation of keratinocyte proliferation and differentiation, maintenance of the epidermal barrier and inflammation (Frateschi et al., 2011).

Skin barrier disruption increases the pH in the epidermis and subsequently increases activity of serine proteases (Hachem et al., 2006; Demerjian et al., 2008). PAR2 activation by a specific activating peptide was shown to delay barrier recovery, accelerate cornification and inhibit lamellar body (LB) secretion after tape-stripping in hairless mice (Hachem et al., 2006; Jeong et al., 2008). In another mouse model, acute barrier disruption caused terminal differentiation of keratinocytes in conjunction with caspase-14, and this differentiation was...
decreased by treatment with serine protease inhibitors (De-
merjian et al., 2008). This event was shown to be regulated by
PAR2-derived cytoskeletal and plasma membrane dynamics
(Roelandt et al., 2011). In PAR2− mice, after tape-stripping, the
skin barrier recovered with enhanced LB secretion (Hachem
et al., 2006; Jeong et al., 2008; Roelandt et al., 2011). Also the
transgenic mice, Grh3 mice, displayed increased expression of
PAR2 and exhibited phenotypes characterized by scaly skin,
hyperplastic epidermis and scratching behavior (Frateschi et
al., 2011).

PAR2 is also correlated with inflammatory responses. PAR2
activation increased the secretion of IL-8, NF-κB-dependent
intercellular cell adhesion molecule-1 (ICAM-1) and prosta-
glandin E2 (PGE2) (Hou et al., 1998, Vergnolle et al., 1998,
Buddenkotter et al., 2005).

We examined the effect of NDGA as a PAR2 antagonist
on the recovery of skin barrier in atopic dermatitis. NDGA is
a natural herbal antioxidant found in the creosote bush, Lar-
rea tridentate (He et al., 2004). NDGA is well known as li-
poxygenase inhibitor and anti-tumor agent (Eads et al., 2009;
Mahajan et al., 2011; Rahman et al., 2011). Notably, NDGA
inhibits the intracellular Ca2+ influx after treatment with trypsin
or PAR2-activating peptide (SLIGKV-NH2). In an oxazolone-in-
duced atopic dermatitis model using hairless mice, we studied
whether NDGA affects keratinocyte differentiation and inflam-
mation by inhibiting PAR2 activation.

MATERIALS AND METHODS

Cell culture

Immortalized human keratinocyte cell line, HaCaT, cells
were purchased from Amore Pacific (Korea). The cells were
generally cultured in high glucose DMEM with FBS 10%, 100
units/ml of penicillin and 100 μg/ml of streptomycin (WelGENE
Inc., Korea). Cells were incubated at 37°C in a humidified at-
mosphere of 5% CO2 and 95% air.

Normal human epidermal keratinocytes (NHEKs) from
neonatal origin were purchased from Invitrogen (USA). The
NHEKs were used until passage number 5. The cells were cul-
tured in EpiLife medium with 60 μM calcium and human ker-
atinocyte growth supplement (Invitrogen, USA) and incubated
at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Measurement of intracellular Ca2+

HaCaT cells were seeded on a 96-well plate at 4×104 cells/
well. After 24 hours, media were changed with 2 μM Fluo-4,
AM (Invitrogen, USA), 0.02% Pluronic F-127 (Invitrogen, USA)
and 2.5 mM Probenecid (Sigma, USA) in Hank’s balanced
salts solution (HBSS, Sigma, USA) containing 20 mM HEPES
(Sigma, USA) for 30 minutes at 37°C and protected from
light. The cells were stabilized for 15 minutes at room
temperature during shaking. Then, NDGA (Sigma, USA) were
pretreated before activation of PAR2. After 30 minutes, 20
μM of PAR2-activating peptide (SLIGKV-NH2, Bachem, Swit-
zerland) or 2 μM of trypsin (Sigma, USA) was added with
Flowstation 3 (Molecular Devices, USA), and intracellular Ca2+
was simultaneously measured using an excitation wavelength
of 485 nm and an emission wavelength of 525 nm at room
temperature. The data were analyzed with SoftMax® Pro (Mo-
olecular Devices, USA), and the mobilization of intracellular
Ca2+ was calculated as the minimum value subtracted from
the maximum value.

Measurement of secreted IL-8 chemokine

HaCaT cells were seeded on 96-well plate at 4×104 cells/
well. After 24 hours, cells were starved with DMEM without
FBS for 12 hours. NDGA were pretreated for 30 minutes. Then,
100 μM of SLIGKV-NH2 was added and cultured for 24 hours. Supernatants were harvested and analyzed with Human
CXCL8/IL-8 DuoSet kit (R&D Systems, USA). Absorb-
ance was measured at 450 nm and corrected at 540 nm using
 Infinite® M200 (TECAN, Switzerland).

Measurement of TARC level

HaCaT cells were seeded on a 96-well plate at 3×104 cells/
well. After 24 hours, cells were starved using DMEM without
FBS for 12 hours. Inflammatory responses were induced by
treating with both INF-γ and TNF-α (ProSpec protein spe-
cialists, Korea) at 10 ng/ml for 24 hours. Then, NDGA was
introduced for 72 hours. Dexamethasone (Sigma, USA) the
positive control was also treated with 100 μg/ml. Superna-
tants were harvested and analyzed with Quantikine® Human
CCL17/TARC immunoassay kit (R&D systems, USA). Absorb-
ance was measured at 450 nm and corrected at 540 nm using
 Infinite® M200.

Western blot

The cells were treated with ice-cold lysis buffer, consisting of
RIPA solution (Noble Bio, Korea), protease inhibitor cock-
tail (Sigma, USA) and 1 mM phenylmethanesulfonl fluoride
(PMSF, Sigma, USA) for 60 minutes. The lysates were soni-
cated and centrifuged at 13,000 × g and 4°C for 20 minutes. Then, supernatants were harvested and protein concentration
was normalized. The protein samples were stored at -20°C.
The samples were mixed with LDS sample buffer and sample
reducing buffer (Invitrogen, USA) and boiled at 95°C for 5
minutes. The reduced samples were loaded on NuPAGE®
10% Bis-Tris gel and run with MOPS SDS running buffer (In-
vitrogen, USA). Then, the proteins were transferred to polyvi-
ylidene fluoride transfer membrane (PVDF membrane, PALL
corporation, USA). The membrane was blocked with 5% skim
milk in TBS-T buffer (0.1% Tween® 20, 100 mM NaCl and 10
mM Tris-HCl, pH 7.5) and then probed using mouse mono-
clonal anti-involucrin antibody (1/1,000, Abcam, UK), sheep
polyclonal anti-ICAM-1 antibody (1/1,000, R&D Systems,
USA), mouse monoclonal anti-β-actin antibody (1/10,000, Sig-
a, USA), donkey anti-sheep IgG antibody (1/5,000, Bethyl
Laboratories, Inc., USA) and goat anti-mouse IgG antibody
(1/10,000, Bio-Rad, USA). Bands were detected with WEST-
ZOL® plus Western Blot Detection system (NIRON Biotechnol-
ology, Korea) and visualized with G : Box chemi (SYNGENE,
USA). Densitometric analysis was performed using Image J
software. The band density was normalized with β-actin inten-
sity.

Animal study

Female hairless mice (SKH-1), aged 6-11 weeks old, were
purchased from HOSHINO laboratory animals (Japan) and
fed mouse diet and water ad libitum. All animal procedures
were approved by the Institutional Animal Care and Use Com-
mitees of Gyeonggi Bio Center and performed in accordance
with their guidelines. The following procedures made refer-
ence to those used by Man et al. (2008). All animals were sensitized with a single topical application of 10% oxazolone (100 μl/mouse) to the dorsal region except for the normal healthy mice group. To induce atopic dermatitis, 150 μl oxazolone 0.5% was topically applied with acetone: olive oil in a 4:1 (v/v) ratio twice daily for 9 days. After 1 day, TEWL was measured using VapoMeter (Defini Technologies, Inc., USA). The mice were regrouped using TEWL levels. Then, 100 μl NDGA was applied with vehicle (propylene glycol: ethanol: water in a 5:3:2 (v/v/v) ratio) twice daily for an additional 5 weeks. Then one hour after NDGA application, oxazolone 0.5% was applied once daily. Dexamethasone 0.05% was used as positive control. TEWL was measured before and after (Day 0) oxazolone-induced atopic dermatitis and during NDGA treatment (Days 10, 22, and 26). Recovery of TEWL was analyzed with following formula: \[
\frac{\text{TEWL of normal group at indicated time}}{\text{TEWL after inducing atopic dermatitis - average TEWL of normal group at indicated time}} \times 100
\]
(Yun et al., 2011). TEWL data were measured four times in each mouse and the TEWL average of each mouse was used as a sample.

Measurement of serum IgE level

Retro orbital sinus blood samples were collected with Mini-Collect® Tube (Greiner Bio-One, Austria) before and after oxazolone-induced atopic dermatitis and again on the final day (Day 27). Collected blood samples were left at room temperature for over 30 minutes to clot. The samples were centrifuged at 4°C and 10,000 rpm for 10 minutes. Then, supernatants were stored at -80°C. Serum IgE concentration was diluted 1/40 with reagent diluent buffer and determined with Mouse IgE ELISA Quantitation kit (Bethyl Laboratories, Inc., USA) following instructions provided by the manufacturer. Absorbance was measured at 450 nm and corrected at 540 nm using Infinite® M200.

Statistics

All data are given as mean ± SEM. Statistical analyses for in vitro studies were performed by t-test using Excel (Microsoft, USA). The data of animal study were analyzed by one-way analysis of variance (ANOVA, SPSS, Inc., USA) followed by post hoc Duncan’s multiple range test to compare differences among groups. Significance was defined as p-value <0.05.

RESULTS

NDGA inhibited PAR2-induced intracellular Ca\(^{2+}\) influx in keratinocytes

PAR2 activation induces intracellular Ca\(^{2+}\) mobilization. HaCaT keratinocytes were treated with SLIGKV-NH\(_2\) or trypsin to induce PAR2 activation, followed by several concentrations of NDGA treatment in order to induce changes in intracellular Ca\(^{2+}\) concentration. The chemical structure of NDGA is shown in Fig. 1. NDGA led to decreased SLIGKV-NH\(_2\) (20 μM) and trypsin- (2 unit/m) induced intracellular Ca\(^{2+}\) mobilization in a dose-dependent manner (Fig. 2). 6.25 μM, 12.5 μM, 25 μM and 50 μM of NDGA treatment inhibited intracellular Ca\(^{2+}\) mobilization in HaCaT keratinocytes by 22%, 42%, 79% and 92% respectively when stimulated by SLIGKV-NH\(_2\) and by 24%, 45%, 80% and 98% respectively when stimulated by trypsin relative to DMSO control (Fig. 2B). Calcium signals generated by HaCaT keratinocytes through release of Ca\(^{2+}\) were significantly reduced by NDGA.

NDGA decreased PAR2-induced inflammatory responses in keratinocytes

PAR2 activation induces inflammatory responses. HaCaT keratinocytes were treated with SLIGKV-NH\(_2\) or TNF-α/INF-γ to induce inflammatory responses. They were simultaneously exposed to various concentrations of NDGA. 100 μM of SLIGKV-NH\(_2\) raised the secretion of IL-8. NDGA was introduced at 1.25 μM, 2.5 μM, 5 μM, 10 μM and 20 μM. The secreted IL-8 level decreased significantly in a dose-dependent manner (Fig. 3A). 25 μM of SLIGKV-NH\(_2\) stimulated ICAM-1 expression, and 0.5 μM and 2 μM of NDGA inhibited ICAM-1 expression (Fig. 3C).

![Fig. 1. Chemical structure of NDGA.](www.biomolther.org)

![Fig. 2. Mobilization of intracellular Ca\(^{2+}\) was decreased by pretreating with a PAR2 antagonist, NDGA. HaCaT keratinocytes were treated with various concentrations of NDGA, from 0.625 μM to 20 μM. After 30 minutes, PAR2-activating peptide, SLIGKV-NH\(_2\), or trypsin was added to activate PAR2, and the mobilization of intracellular Ca\(^{2+}\) was measured immediately by Flexstation 3. (A) Intracellular Ca\(^{2+}\) immediately increased with the addition of SLIGKV-NH\(_2\). The mobilization of intracellular Ca\(^{2+}\) was inhibited by pretreating with NDGA, in a dose-dependent manner. (B) NDGA decreased intracellular Ca\(^{2+}\) influx induced by both SLIGKV-NH\(_2\) and trypsin in HaCaT keratinocytes. Data are shown as Mean ± SEM, n=2. *p<0.05, ** (or †) p<0.01, *** (or ‡) p<0.001, compared with the control. RFU: relative fluorescence unit.](www.biomolther.org)
mokine (TARC) was induced by exposure to TNF-α and IFN-γ at 10 ng/ml, 1.25 μM, 2.5 μM, 5 μM, 10 μM and 20 μM of NDGA decreased the secreted TARC level, though not statistically significantly, in a dose-dependent manner (Fig. 3B). Dexamethasone (steroid) was used as positive control and decreased TARC level significantly at 250 μM.

NDGA inhibited PAR2-induced terminal differentiation in keratinocytes

PAR2 activation induced terminal differentiation of keratinocytes. In differentiating keratinocytes, many proteins such as involucrin, loricrin and filaggrin are upregulated (Steven and Steinert, 1994). We investigated whether NDGA decreased involucrin protein expression by inhibiting PAR2 activation in HaCaT keratinocytes and NHEKs. In HaCaT keratinocytes, treatment with 100 μM SLIGKV-NH₂ for 72 hours showed a 3.4-fold increase in involucrin expression compared with control. NDGA exposure at 0.5 μM, 2 μM and 10 μM inhibited involucrin expression by 1.3-, 0.3- and 0.9-fold, respectfully, compared with treatment of SLIGKV-NH₂ alone (Fig. 4A). In NHEKs, 100 μM of SLIGKV-NH₂ was introduced for 120 hours and upregulated involucrin protein level 1.4-fold. SLIGKV-NH₂ was treated with 0.01 μM, 0.05 μM and 0.2 μM of NDGA and all doses except 0.01 μM dose-dependently downregulated the involucrin expression (Fig. 4B).

NDGA effects on oxazolone-induced atopic dermatitis in hairless mice

To investigate whether NDGA could recover skin barrier in atopic dermatitis in vivo, we induced atopic dermatitis in hairless mice using oxazolone 0.5% topically to dorsal skin for 9 days, followed by NDGA. Skin barrier recovery was checked by dorsal TEWL change. Dexamethasone 0.05% was used as a positive control. At 10 days after NDGA treatment (0.01%, 0.1% and 1%), skin barrier was recovered at 18%, 26% and 7% respectively, while vehicle treatment scarcely affect...
DISCUSSION

AD is a chronic inflammatory skin disease which occurs mainly in childhood and is a pervasive health problem in developed countries (Kang et al., 2008). Both abnormal inflammatory response and defective skin barrier are characteristic of AD (Jin et al., 2009). Barrier disruption increases the invasion of allergens, such as dust mite waste, pollen and pet dander and causes more severe inflammation (Elias and Steinhoff, 2008). This cycle comprises the "outside-inside-outside" model of AD pathogenesis (Elias and Steinhoff, 2008). PAR2 correlates with inflammatory responses and differentiation of keratinocytes. The proliferation and differentiation of keratinocytes in the epidermis control barrier permeability and homeostasis. The aim of this study is to find a new PAR2 antagonist to interfering with PAR2 activation, and consequently to halt symptoms of atopic dermatitis. Serine protease inhibitors, such as aprotinin and t-AMCHA, are known for inhibiting PAR2 activation by blocking cleavage of the N-terminus and subsequent inhibition of PAR2-activating peptide (He et al., 2004). We found that NDGA decreased intracellular Ca\(^{2+}\) mobilization induced by SLIGKV-NH\(_2\) and trypsin in HaCaT keratinocytes (Fig. 2). Our results indirectly indicated that PAR2 activation can be specifically inhibited by NDGA. Consequently, we have shown that NDGA inhibits PAR2-induced inflammation and keratinocyte differentiation.

PAR2 activation is known to increase the secretion of IL-8 and NF-κB-dependent ICAM-1 expression (Hou et al., 1998; Vergnolle et al., 1998). Additionally, PAR2 activation stimulates the mRNA expression of IL-1α, IL-8, TNF-α and human beta defensin (hBD)-2 in keratinocytes (Lee et al., 2010). Recent studies have also revealed the importance of PAR2 in itching and pain sensations. In rat and mouse DRG neurons, PAR2 was shown to be associated with long-lasting thermal and mechanical hyperalgesia by mediating the transient receptor potential vanilloid 4 ion channel (Grant et al., 2007; Jeong et al., 2008). We showed that NDGA inhibited PAR2-induced inflammation. SLIGKV-NH\(_2\) treatment increased IL-8 and ICAM-1 expression in HaCaT keratinocytes. NDGA treatment decreased IL-8 and ICAM-1 expression in a dose-dependent manner. Also, NDGA treatment decreased TARC secretion induced by TNF-α and IFN-γ in HaCaT keratinocytes (Fig. 3).
PAR2 activation induces the abnormal differentiation of keratinocytes. In previous studies, epidermal barrier disruption by repetitive tape-stripping caused a pH rise and an increase in serine protease activity. Serine protease was shown to cleave the PAR2 N-terminus, activating PAR2. PAR2 activation induced abnormal differentiation by stimulating cornification and inhibiting lamellar body secretion, thus delaying barrier recovery (Hachem et al., 2006, Demerjian et al., 2008; Jeong et al., 2008). To confirm the effects of NDGA on keratinocyte differentiation, we examined the expression of involucrin in HaCaT keratinocytes and NHEKs. NDGA decreased the expression of involucrin induced by SLGKV-NH2 in both HaCaT keratinocytes and NHEKs (Fig. 4).

To determine the effects of NDGA on atopic dermatitis, we used an oxazolone-induced atopic dermatitis model. Oxazolone-induced atopic dermatitis was checked by measuring TEWL. Basal TEWL was about 20 g/m²/h, and after AD induction TEWL was over 70 g/m²/h. Topical treatment of NDGA decreased TEWL, and NDGA had the best effect on barrier recovery at day 10 (Fig. 5A). Also treatment with NDGA at concentrations of 0.01% and 1% reduced serum IgE level by 22%, 5% and 24%, respectively, compared with vehicle at day 27 (Fig. 5B). From these results, we concluded that NDGA could affect barrier recovery in atopic dermatitis without inducing weight loss.

In summary, NDGA blocked the PAR2-mediated signal pathway and promoted skin barrier recovery in atopic dermatitis. Our results suggest that NDGA is a PAR2 antagonist and may be a potential therapeutic agent for atopic dermatitis. For further study, we recommend examining the effects of NDGA on atopic dermatitis by using NC/Nga mice, a mice strain that spontaneously develops atopic dermatitis.

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