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Reactive nitrogen species formation in eosinophils and imbalance in nitric oxide metabolism are involved in atopic dermatitis-like skin lesions in NC/Nga mice

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Abstract

Nitric oxide (NO) and reactive nitrogen species (RNS) have been implicated in the pathogenesis of inflammatory diseases. However, the involvement of NO and RNS in atopic dermatitis (AD), a pruritic inflammatory skin diseases, is not fully understood. In this study, we investigated the contribution of NO and RNS to the development of AD-like skin lesions in NC/Nga mice, an animal model for human AD. AD-like skin lesions were observed in NC/Nga mice kept under conventional conditions but not in specific pathogen-free conditions. The expression of inducible NO synthase (iNOS) and endothelial NOS (eNOS) proteins was upregulated in the dermal lesions, and that of neuronal NOS (nNOS) was downregulated in the epidermal lesions of the skin. Although the concentrations of NO_2^- and NO_3^- were lower, protein-bound nitrotyrosine content was significantly increased in the skin lesions. Immunohistochemical localization of nitrotyrosine was observed in almost all eosinophils. These results suggest that RNS formation in eosinophils and imbalance of NO metabolism are involved in the pathogenesis of AD-like skin lesions in NC/Nga mice.

Keywords: *Nitric oxide, reactive nitrogen species, nitric oxide synthase, nitrotyrosine, atopic dermatitis, NC/Nga mice*

Abbreviations: *AD, atopic dermatitis; Df, Dermatophagoides farinae*; Dp, *Dermatophagoides pteronyssinus*; DNP, dinitrophenyl; EDTA, ethylene diamine tetraacetic acid; eNOS, endotherial nitric oxide synthase; H_2O_2, hydrogen peroxide; HPLC, high performance liquid chromatography; HPLC-ECD, high performance liquid chromatography with electrochemical detection; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NO_2, nitrogen dioxide; NOS, nitric oxide synthase; NOx, nitrite and nitrate; $O_2^{\cdot-}$, superoxide anion; $ONOO^-$, peroxynitrite; PBS, phosphate-buffered saline; RNS, reactive nitrogen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS-T, Tris-buffered saline containing 0.5% Tween 20

Introduction

Nitric oxide (NO) plays significant roles in the development of inflammation [1–3]. It is produced from L-arginine by three different isoforms of NO synthase (NOS): constitutive NOS [neuronal NOS (nNOS) and endothelial NOS (eNOS)] and inducible NOS (iNOS). While constitutive NOS (eNOS and nNOS) produces low levels of NO for homeostatic functions, iNOS generates much higher levels of NO in inflammatory and pathological conditions [1–3]. NO is physiologically converted to NO_2^- and NO_3^-, major oxidative products of NO metabolism, by different oxidation reactions [4]. In inflammatory conditions, NO produced by iNOS

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reacts with superoxide anion ($O_2^{\cdot-}$) generated nearby to yield peroxynitrite ($ONOO^-$), a powerful oxidizing species [5,6]. $ONOO^-$ causes oxidation of DNA, lipids and proteins, and most notably, nitration of free and protein-bound tyrosine. The formation of nitrotyrosine was initially considered to the footprint of $ONOO^-$ [5,6]. Alternatively, nitrogen dioxide (NO_2) derived from the catalytic oxidation of NO_2^- by hydrogen peroxide (H_2O_2) with peroxidase can also lead to tyrosine nitration [7]. Thus, nitrotyrosine is a indicator of the formation of reactive nitrogen species (RNS) rather than a specific marker of $ONOO^-$. The formation of nitrotyrosine has been demonstrated in various human diseases such as asthma, atherosclerosis, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease [8].

Atopic dermatitis (AD) is a highly pruritic, chronic, relapsing and inflammatory skin disease with unknown etiology. Most patients with AD show elevation of eosinophils in the peripheral blood and increases in serum IgE levels as the clinical features [9,10]. It has been speculated that iNOS is upregulated in AD based on the immunohistochemical detection of iNOS in skin lesions of AD patients [11] and in AD-like skin lesions induced by repeated application of hapten in NC/Nga mice [12]. However, there is no agreement on the participation of NO in the pathogenesis of AD because of the discrepancy between the NOx levels in the serum [13] versus the urine [14] of AD patients. Moreover, the involvement of RNS formation in AD remains unclear.

NC/Nga mice, originated from Japanese fancy mice (Nishiki-Nezumi) and established as an inbred strain by Kondo (Nagoya University, Nagoya, Japan), have certain characteristics such as high susceptibility to irradiation and to anaphylactic shock by ovalbumin [15,16]. In addition, Matsuda et al. have demonstrated that NC/Nga mice are available as an animal model for human AD [17]. NC/Nga mice spontaneously suffer severe skin lesions similar to those in human AD, characterized by hemorrhage, edema, erythema, dryness, infiltration of inflammatory cells, acanthosis and hyperkeratosis when kept in conventional conditions but not when kept in specific pathogen-free conditions [17,18]. Therefore, in this study, we investigated the contribution of NO and RNS to the development of AD-like skin lesions in NC/Nga mice.

Materials and methods

Animals

Male 4-week-old NC/Nga mice were purchased from Japan SLC (Shizuoka, Japan). The NC/Nga mice were divided into two groups. One group was kept under specific pathogen-free conditions and the other under non-sterile conventional conditions in which dust was not removed due to air-uncontrolled without filtration. All animal experiments were performed according to the standards of the animal care guidelines of Kanazawa University.

Preparation of skin samples

Mice were sacrificed under light ether anesthesia. The dorsal skin was excised and the subcutaneous fat and blood vessels were carefully removed.

The skin samples were homogenized in five volumes of homogenate buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA) and complete protease inhibitor mixture tablets (Roche, Mannheim, Germany)], and centrifuged at 1000*g* for 10 min at 4°C, and the supernatant fraction was stored for subsequent analysis. The precipitate was washed three times with homogenate buffer and solubilized in homogenate buffer containing 1% Triton X-100. Protein concentrations were determined using a BCA protein assay reagent kit (PIERCE, Rockford, IL) with bovine serum albumin as a standard.

Histological analysis

Excised dorsal skin was fixed with 10% neutral-buffered formalin and embedded in paraffin. Tissue sections (3–5 μm thick) were stained with hematoxylin and eosin and examined under a light microscope (Olympus IX-70; Tokyo, Japan).

Measurement of serum IgE

Blood was collected from the retro-orbital plexus of mice under light ether anesthesia. Serum samples were stored at −30°C. Serum IgE levels were estimated using a mouse IgE ELISA kit (Amersham Pharmacia Biotech, Buckinghamshire, England) according to the manufacturer's instructions. The limit of detection of IgE was 1.7 ng/ml.

Immunohistochemistry

Immunostaining was performed as reported previously [19]. Deparaffinized skin sections were treated with 3% H_2O_2 in methanol to inactivate endogenous peroxidases, and then incubated with 5% normal goat serum. Before each step, the sections were washed with 3 changes of Tris-buffered saline (TBS). The specimens were incubated overnight at 4°C with polyclonal rabbit antibodies against iNOS, eNOS or nNOS (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) or a polyclonal rabbit anti-nitrotyrosine antibody (1:800, Upstate Biotechnology, Lake Placid, NY). Then the specimens were treated with goat anti-rabbit immunoglobulin conjugated with peroxidase labeled-dextran polymer (Dako, Carpinteria, CA) for 1 h at room temperature. Visualization of the enzyme

reaction was performed with 3, 3′-diaminobenzidine tetrahydrochloride (Dako) as a substrate, and counterstaining was carried out with hematoxylin. For the detection of eosinophils, the sections were double-stained with Luxol-fast-blue as described previously [19–21]. As a negative control, rabbit non-immune immunoglobulin (Dako) was used. The specificity of staining for nitrotyrosine immunohistochemistry was verified by pre-incubation of the anti-nitrotyrosine antibody with 10 mM nitrotyrosine or by incubation of tissue sections with 100 mM sodium dithionite to convert nitrotyrosine to aminotyrosine.

Measurement of NO_2^- *and* NO_3^-

Serum and the supernatant fractions of skin samples were filtrated with a 10 kDa molecular cut-off filter unit (Millipore, Bedford, MA) to remove proteins and other debris. The concentrations of NO_2^- and NO_3^- in the filtrates were measured using a high performance liquid chromatography (HPLC)-Griess system[22]. In brief, 10 μl of sample was injected into the system, in which NO_2^- and NO_3^- were separated on a reverse phase column (NO-PAK, Eicom, Kyoto, Japan) and NO_3^- was reduced to NO_2^- by passage through a reduction column (NO-REP, Eicom). NO_2^- was measured as the azo dye compound formed by the Griess reaction using a UV–VIS detector at 540 nm.

Western blot analysis

The supernatant fractions of skin samples (70 μg/well) were subjected to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% nonfat dry milk in TBS containing 0.5% Tween 20 (TBS-T) for 1 h at room temperature, and then reacted at 4°C overnight with polyclonal rabbit antibodies against iNOS, eNOS or nNOS (1:500, Santa Cruz Biotechnology). After washing with TBS-T, the membrane was incubated with a horseradish peroxidase (HRP) -conjugated goat anti-rabbit immunoglobulin antibody (1:4000, Dako) for 1 h at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence Western blot detection system (Perkin-Elmer, Boston, MA). The density of the specific bands was quantified using NIH image shareware.

Measurement of nitrotyrosine

Protein-bound nitrotyrosine concentrations were measured in the supernatant fractions and Triton X-100-solubilized fractions of skin samples. Protease digestion was performed as reported previously [23]. Eight-fifths-fold volumes of acetonitrile were added to a protein suspension (3 mg protein), which was vortexed for 3 s, and centrifuged at 2000*g* for 5 min at 4°C. The precipitate was dissolved into 0.5 ml of 0.1 M sodium acetate buffer, pH 7.2, and reprecipitated with 0.8 ml acetonitrile as describe above. The washing procedure was repeated as above to remove residual NO_2^-, NO_3^- and other contaminants. The final pellet was dispersed into 0.5 ml of 0.1 M sodium acetate buffer, pH 7.2 containing 0.9 mg of dialyzed pronase (Roche) and incubated for 16 h at 50°C. The digestion products were centrifuged at 2000*g* for 10 min at 4°C, and the supernatant was filtrated through a 10 kDa molecular cut-off filter unit (Millipore). Then, 50 μl of the filtrate was injected into the HPLC apparatus with electrochemical detection (HPLC-ECD; Eicom) to quantify nitrotyrosine [24]. A SC-5ODS column (3 × 150 mm, 5 μm; Eicom) was used as an analytical column. The mobile phase was 2% acetonitrile containing 0.2 M sodium dihydrogenphosphate and 0.5 mg/l EDTA. The flow rate was 0.5 ml/min and the column temperature was kept at 25°C. Nitrotyrosine was reduced to aminotyrosine using a through-type electrolytic cell (−900 mV) after separation with the column. The resulting products were analyzed with an amperometric electrochemical detector (+300 mV). Tyrosine was quantified by HPLC using a Spherisorb ODS2 column (4.6 × 150 mm, 5 μm; Waters, Milford, MA) with 93% 50 mM potassium phosphate and 7% methanol as the mobile phase at a flow rate of 1.0 ml/min [20,25]. Results are expressed as the mole ratio of nitrotyrosine to tyrosine.

Measurement of serum levels of mite allergen-specific IgG1 and IgE

The serum levels of mite allergen-specific IgG1 and IgE were measured by ELISA. Briefly, microtiter plates were coated with 2 μg/100 μl/well of *Dermatophagoides farinae* (Df) or *Dermatophagoides pteronyssinus* (Dp) crude extracts. After blocking with phosphate-buffered saline (PBS) containing 5% FBS and washing with PBS containing 0.05% Tween 20, appropriate dilutions of serum samples in PBS containing 5% FBS were added to the plate and incubated overnight. The IgG1 or IgE antibodies bound to the plates were quantitated using biotinylated anti-mouse IgG1 (Zymed, San Francisco, CA) or IgE (LO-ME-2 Biotin; Cosmobio, Tokyo, Japan), HRP-streptavidin conjugate (Zymed) and TMB One-Step substrate (Dako). The level of IgG1 or IgE was calculated by comparison with a reference curve prepared with mouse IgG1 against anti-dinitrophenyl (DNP) (MADNP-1; Cosmobio) or mouse IgE against anti-DNP (Seikagaku Corp., Tokyo, Japan). Briefly, plates were coated with DNP-conjugated OVA at the same concentration as the mite crude extracts. After overnight incubation at 4°C, the plates were washed and blocked and developed as described above.

Statistical analysis

All data are expressed as mean ± SD. The significance of the differences between groups was analyzed by one-way analysis of variance, and $P < 0.05$ was considered significant.

Results

General condition of the NC/Nga mice

From 8 weeks of age, NC/Nga mice kept under conventional conditions started to scratch their faces, necks and ears, and apparently showed several symptoms of dermatitis, such as hemorrhage, eczema, erythema and dryness, whereas the mice kept under specific pathogen-free conditions showed no such symptoms. The severity of the skin lesions was exacerbated with age, resulting in heavy inflammation, acanthosis and hyperkeratosis. The development of skin lesions was accompanied by an increase in the serum IgE concentration, which reached 9.79 ± 1.89 μg/ml ($n = 9$) at 20 weeks of age. By contrast, the mice kept under specific pathogen-free conditions showed no elevation of the IgE level.

NO_2^- and NO_3^- concentrations in serum and skin tissue

The concentrations of serum NO_2^- and NO_3^- in the mice kept under specific pathogen-free conditions were 0.634 ± 0.142 μM ($n = 9$) and 6.94 ± 2.14 μM ($n = 9$), respectively. In the serum of mice kept under conventional conditions, the NO_2^- and NO_3^- concentrations were significantly higher than those in the mice kept under specific pathogen-free conditions, 1.18 ± 0.506 μM ($n = 9$, $P < 0.01$) and 13.28 ± 3.21 μM ($n = 9$, $P < 0.001$), respectively (Figure 1A). The NO_2^- and NO_3^- concentrations in the normal skin of mice kept under specific pathogen-free conditions were 0.120 ± 0.087 nmol/mg protein ($n = 7$) and 1.41 ± 0.679 nmol/mg protein ($n = 7$), respectively. Significantly lower concentrations of cutaneous NO_2^- (0.084 ± 0.070 nmol/mg protein, $n = 9$, $P < 0.05$) and NO_3^- (0.511 ± 0.150 nmol/mg protein, $n = 9$, $P < 0.001$) were detected in the mice kept under conventional conditions (Figure 1B).

Protein expression of NOS isoforms

The expression of NOS isoforms was investigated by Western blot analysis (Figure 2A and B). The protein expression of iNOS ($n = 6$, $P < 0.05$) and eNOS ($n = 6$, $P < 0.01$) was significantly increased in the skin lesions of mice kept under conventional conditions. In contrast, the expression of nNOS tended to decrease in the skin lesions compared to the normal skins of mice kept under specific pathogen-free conditions ($n = 6$, $P = 0.211$).

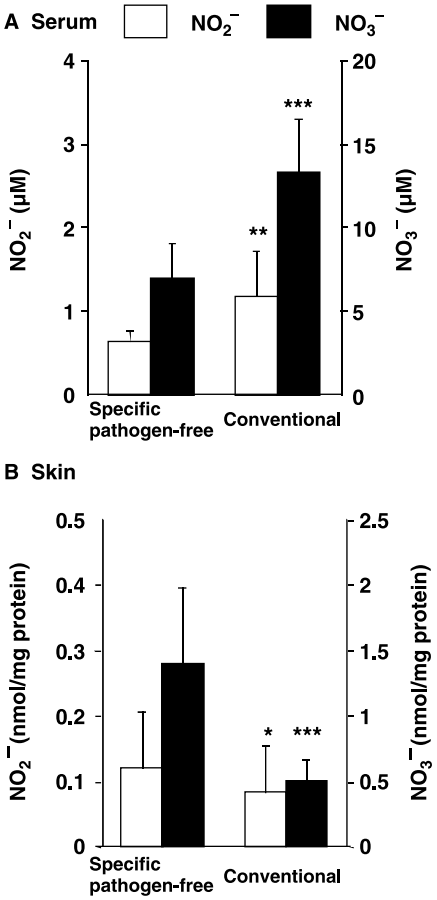

Figure 1. NO_2^- and NO_3^- concentrations in the serum and the skin of NC/Nga mice kept under specific pathogen-free and conventional conditions. The concentrations of NO_2^- (open bars) and NO_3^- (solid bars) in the serum (A) and the skin (B) were analyzed using an HPLC-Griess system. Results are shown as the mean ± SD of seven to nine animals/group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with specific pathogen-free.

Immunohistochemistry for NOS isoforms and nitrotyrosine

The localization of NOS isoform proteins was investigated by immunohistochemical methods. Cells immunostained for iNOS were localized in inflammatory cells infiltrated into the dermal lesions of mice kept under conventional conditions (Figure 3A), but were not detected in the normal skins of mice kept under specific pathogen-free conditions (Figure 3B). Increased immunostaining for eNOS was seen in the vascular endothelium in the dermal lesions (Figure 3C) compared with the level of staining in normal skins (Figure 3D). Immunostaining for nNOS was detected in keratinocytes and sebaceous gland cells (Figure 3F). Less immunostaining of nNOS was found in the basal layer of the hyperplastic epidermis of skin lesions (Figure 3E). Immunostaining was not observed after incubation of the sections of skin lesions with non-immune rabbit immunoglobulin as a primary antibody (data not shown). To examine the involvement of RNS formation in AD-like skin lesions, the localization of nitrotyrosine was also

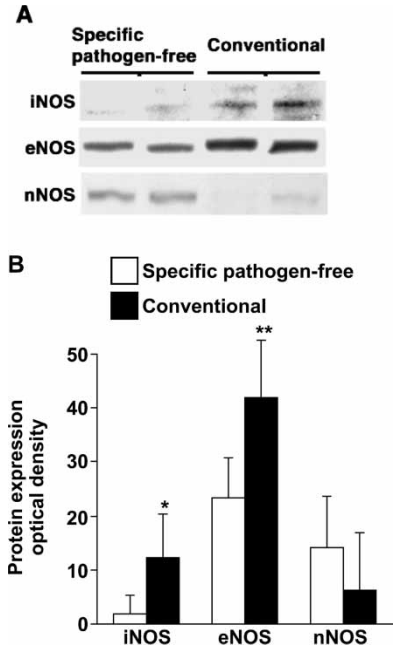

Figure 2. Protein expression of NOS isoforms in the skin of NC/Nga mice kept under specific pathogen-free and conventional conditions. Expression of NOS isoforms was examined by Western blot analysis (A) and quantified densitometrically (B). Results are shown as the mean ± SD of six animals/group. $*P < 0.05$, $**P < 0.01$, compared with specific pathogen-free.

examined. In the skin lesions, strong and diffuse nitrotyrosine staining was observed in the granulocytes in the dermal lesions (Figure 3G). The majority of cells positive for nitrotyrosine were eosinophils, as shown by co-staining with Luxol-fast-blue (Figure 3I and J). In the normal skins, there was little staining for nitrotyrosine (Figure 3H). Most of the cells positive for nitrotyrosine showed immunoreactivity for iNOS, as judged from the investigation of adjacent serial sections. Immunostaining for nitrotyrosine was abolished by preincubation of the anti-nitrotyrosine antibody with 10 mM nitrotyrosine, by treatment of the sections with 100 mM sodium dithionite, or by using non-immune rabbit immunoglobulin as the primary antibody (data not shown).

Nitrotyrosine content

As shown in Figure 4, the cutaneous content of nitrotyrosine in the Triton X-100-solubilized fraction was significantly higher in the skin lesions (26.58 ± 7.87 nmol nitrotyrosine/mmol tyrosine, $n = 7, P < 0.05$) in comparison with that in the normal skins (18.49 ± 3.10 nmol nitrotyrosine/mmol tyrosine, $n = 7$). There was no significant difference between the nitrotyrosine levels in the supernatant fractions of skins from mice kept under conventional (7.52 ± 1.77 nmol nitrotyrosine/mmol tyrosine, $n = 7$, $P = 0.46$) and specific pathogen-free conditions (9.08 ± 5.05 nmol nitrotyrosine/mmol tyrosine, $n = 7$).

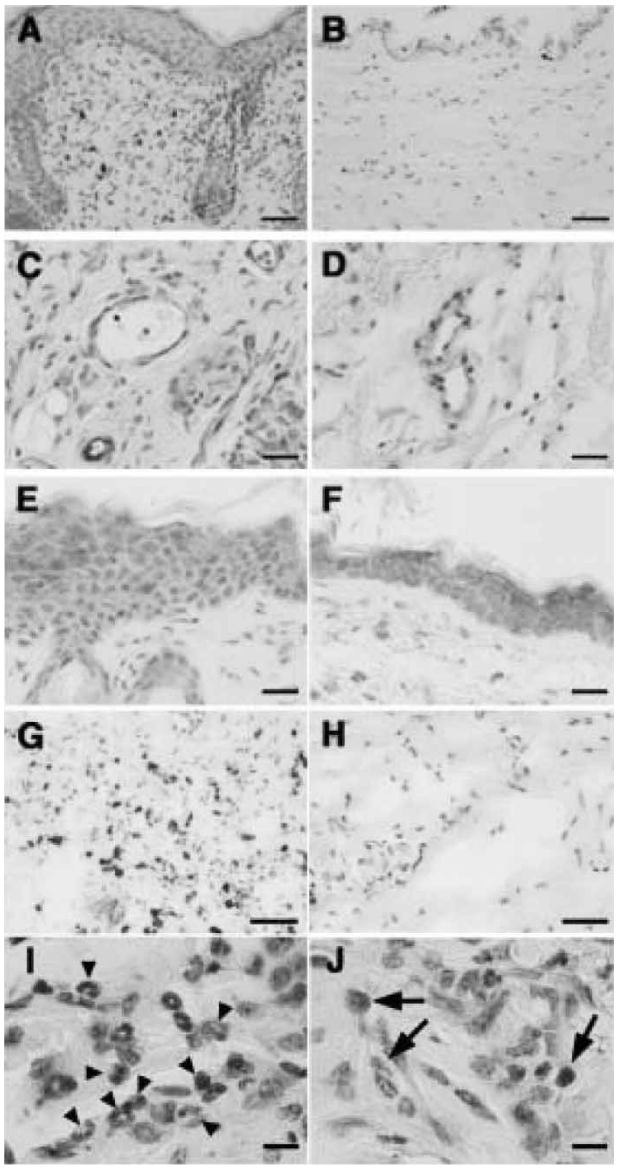

Figure 3. Immunohistochemical staining for NOS isoforms and nitrotyrosine in the skin of NC/Nga mice kept under specific pathogen-free and conventional conditions. Many immunoreactive cells for iNOS were seen in the dermal lesions (A), but were not detected in the normal skins (B). Increased staining for eNOS was seen in vascular endothelial cells of the dermal lesions (C) compared with the level of staining in normal skins (D). Less immunostaining of nNOS was seen in the basal layer of hyperplastic epidermis of skin lesions (E) compared with the normal skins (F). Cells immunostained for nitrotyrosine were seen in the dermal lesions (G) compared with fewer stained cells in normal skins (H). Eosinophils were stained with Luxol-fast-blue (I, arrowheads). Cells positive cells for nitrotyrosine were co-stained with Luxol-fast-blue in the dermal lesions (J, arrows). Bars indicate 10 μm (I and J), 50 μm (C–H) and 100 μm (A and B).

Serum mite allergen-specific IgG1 and IgE

The serum levels of mite allergen-specific IgG1 and IgE were significantly increased in the mice kept under conventional conditions compared to the mice kept under specific pathogen-free conditions (Table I).

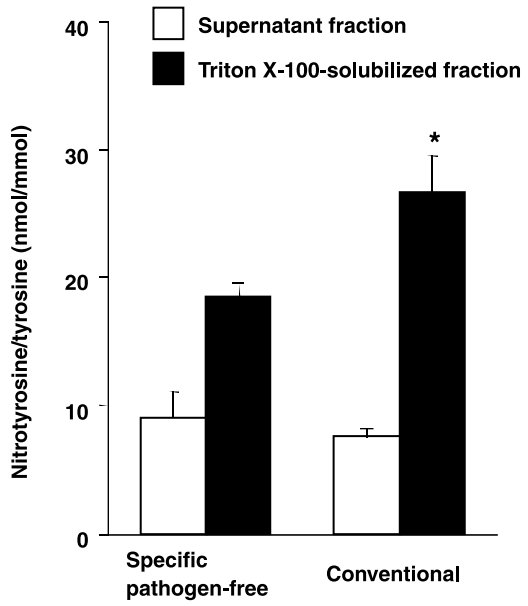

Figure 4. Nitrotyrosine content in the skin of NC/Nga mice kept under specific pathogen-free and conventional conditions. Protein-bound nitrotyrosine in the supernatant fractions (open bars) and the Triton X-100-solubilized fractions (solid bars) was measured by HPLC-ECD after pronase digestion. Results are shown as the mean ± SD of seven animals/group. $^{\star}P < 0.05$, compared with specific pathogen-free.

Discussion

NC/Nga mice have been reported to develop AD-like skin lesions, when kept under conventional conditions but not when kept under specific pathogen-free conditions [17,18]. We demonstrated here that changes in the expression of NOS isoforms, increases in RNS formation and subsequent decreases in NOx (NO_2^- and NO_3^-) occurred in AD-like skin lesions in NC/Nga mice (Figure 5).

An increase in the serum NOx concentration was previously reported in AD patients [13]. In fact, we observed that the concentration of serum NOx was significantly higher in the mice kept under conventional conditions than in the mice kept under specific pathogen-free conditions. Therefore, we expected high concentrations of NOx in the skin lesions. On the contrary, the concentrations of NOx in the skin lesions of mice kept under conventional conditions were lower than the control levels in mice kept under specific pathogen-free conditions, although upregulation of iNOS and eNOS expression was observed in inflammatory cells and vascular endothelial cells, respectively. The decreased concentrations of NOx in the skin lesions may be explained by differences of NO metabolism between the dermis and epidermis. In the dermal lesions, iNOS-generated NO seems to be mainly consumed by RNS formation in eosinophils, where nitrotyrosine is formed. Upregulation of eNOS expression may be responsible, at least in part, for the increase in RNS formation near blood vessels as well as the elevated NOx concentration in the serum in mice kept under conventional conditions. In this regard, it is reasonable to suppose that the increased NO resulting from the upregulation of NOS expression was used for the formation of RNS, leading to a decrease in the NOx concentration in the dermal lesions. Downregulation of nNOS expression in the cells of the basal layer of hyperplastic epidermis appears to cause a decrease in NO production. Reduced NO production in the epidermal lesions may have accounted for a significant share of the total decrease in the NOx concentration in the skin. We thus propose that alteration of NO metabolism is involved in the development of AD-like skin lesions.

In the present study, almost all cells positive for nitrotyrosine were eosinophils in the dermal lesions of mice kept under conventional conditions. This finding is consistent with our finding that nitrotyrosine was significantly increased by solubilization of eosinophils (sedimented by centrifugation [26]) in skin homogenate prepared using Triton X-100. Infiltration and activation of eosinophils is a characteristic feature of AD skin lesions [27,28]. It has been suggested that allergen-specific IgG1and IgE are responsible for the activation and degranulation of eosinophils and the manifestations of allergic diseases [29,30]. In fact, we found that serum levels of mite allergen-specific IgG1 and IgE were significantly increased in the mice kept under conventional conditions, implying the activation of eosinophils that infiltrated into the dermal lesions. Activated eosinophils can generate $O_2^{\cdot-}$, via the activation of NADPH oxidase, and its dismutation product H_2O_2 [31]. Interestingly, we detected the expression of iNOS in most of the cells positive for nitrotyrosine. iNOS-derived NO reacts with O_2^- to form $ONOO^-$, resulting in nitration of tyrosine [5,6]. In addition, eosinophils containing heme peroxidase can induce the nitration of protein tyrosine residues by

Table I. Mite allergen-specific IgG1and IgE levels in serum of NC/Nga mice kept under specific pathogen-free and conventional conditions.

		Mite allergen specific antibody levels (ng/ml)			
Group	*n*	Df-specific IgG1	Dp-specific IgG1	Df-specific IgE	Dp-specific IgE
Specific pathogen-free	8	18.6 ± 9.1	21.6 ± 14.1	1.1 ± 1.9	0.29 ± 0.81
Conventional	6	1390.0 ± 1678.4*	1700.0 ± 1892.9*	7.8 ± 6.8*	8.0 ± 6.8**

The levels of mite allergen-specific IgG1and IgE in serum were measured by ELISA. Results are shown as the mean ± SD. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, compared with specific pathogen-free.

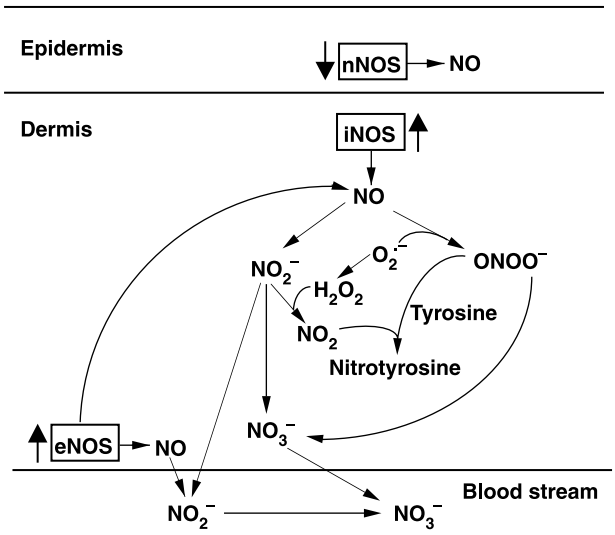

Figure 5. Schematic representation of metabolic pathways of NO in AD-like skin lesions. In the hyperplastic epidermis, downregulation of nNOS expression leads to deceased NO production. In the dermis, upregulation of iNOS and eNOS expression occurs and NO produced subsequently is mainly consumed by RNS formation, resulting in nitrotyrosine formation. The distinct pathways of NO metabolism in the epidermis and dermis may be responsible for the decrease in NOx concentration in AD-like skin lesions.

oxidizing NO_2^- in the presence of H_2O_2 [32]. Previous reports showed that eosinophil-related nitrotyrosine formation in lung tissue and sputum correlates with the pathology of asthma [33,34]. Accumulated evidence shows the involvement of eosinophils in the development of AD [27,28]. Therefore, nitrotyrosine-containing eosinophils, activated during inflammation, may have a key role in the pathogenesis of AD.

We should also consider the roles of other molecules caused by RNS formation because not all of the increased RNS is necessarily used for nitrotyrosine formation. The targets of RNS include not only tyrosine, but also nucleic acid, lipid and amino acids such as cysteine and tryptophan. A previous study demonstrated the generation of 8-nitroguanosine, a product of guanosine nitration, *in vivo* via iNOS-derived NO [35]. S-Nitrosothiols are formed by the reaction of the thiol of cysteine with nitrosating species, such as N_2O_3, following the autooxidation of NO [3]. Additionally, nitrotryptophan [36] and nitrolinoleate [37] can also be formed by a reaction of RNS with tryptophan and linoleic acid, respectively. Whether these products were formed in the present study was not examined, but they may partly contribute to the consumption of NO in the skin lesions.

Constitutive expression of nNOS has been observed in the keratinocytes of the epidermis [2]. Interestingly, we found that nNOS expression tended to be decreased in the skin lesions of mice kept under conventional conditions. Immunohistochemistry also provided consistent results, namely, weaker staining for nNOS in the basal layer of the hyperplastic epidermis. In addition, we did not observe immunostaining for iNOS in the keratinocytes of epidermal lesions. The physiological role of NO in the epidermis is the protection of the skin surface and facilitation of wound healing [2]. Impairment of the barrier function in the affected skin is a common finding in both AD patients [9,10,38] and NC/Nga mice [39]. It results in an increased frequency of the development of bacterial and fungal skin infections among patients with AD [9,10]. A recent report demonstrated the upregulation of nNOS in the keratinocytes during skin repair [40]. The tendency for nNOS to be decreased in AD-like skin lesions may be associated with the impairment of wound healing and of antimicrobial function, resulting in the occurrence and exacerbation of skin barrier dysfunction.

It has been suggested that increased oxidative stress is involved in AD. For instance, AD patients show increased levels of 8-hydroxydeoxyguanosine in their urine as an index of oxidative damage to DNA [14,41]. A recent report showed that protein oxidative damage occurs in the stratum corneum of the skin from AD patients [42]. These reports are supported by our observation that nitrotyrosine was increased in the dermal lesions, because the formation of nitrotyrosine requires the generation of reactive oxygen species as well as NO. Moreover, we demonstrated here that enhancement of oxidative damage took place in the dermis in addition to the epidermis of the skin lesions.

In this communication, we characterized the formation of RNS in eosinophils regarded as activated, and demonstrated the imbalance of NO metabolism in the skin lesions of an animal model for AD. Additional studies with NOS inhibitors or iNOS knockout mice and direct demonstration of the activation of eosinophils will be needed to further clarify the association between NO and the pathogenesis of AD.

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