

Aggravation of atopic dermatitis-like symptoms by consecutive low concentration of formaldehyde exposure in NC/Nga mice

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Abstract: Formaldehyde (FA) has been known to be associated with development of asthma (AS) and atopic dermatitis (AD). In this study, we investigated whether FA inhalation would affect the provocation or exacerbation of AD-like symptoms. Atopic-prone NC/Nga mice were exposed to low (0.2 ppm) and high (1.0 ppm) concentration of FA by inhalation. Combined exposure to low concentration of FA inhalation and topical house dust mite (HDM) stimulation significantly upregulated HDM-induced total plasma IgE and IgG2a production, Th1-, Th2-, Th17-related cytokine as well as COX-2 mRNA expressions in the skin. Interestingly, independent FA inhalation, especially at low concentration

(0.2 ppm), increased the skin mRNA expressions of IL-13, IL-17E/IL-25 and COX-2, even though it failed to induce AD-like skin inflammation. In conclusion, we suggest that increased skin mRNA expressions of IL-13, IL-25/IL-17E and COX-2 by independent low concentration of FA exposure might be a key factor to exacerbate HDM-mediated AD-like skin inflammation.

Key words: atopic dermatitis – formaldehyde – house dust mite – NC/Nga mouse – proallergic cytokines

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Background

Nowadays, many studies pay attention to the association between indoor environmental factors (e.g. mites, microbiological agents and volatile organic compounds) and the increased prevalence of allergic atopic diseases (1–4). Formaldehyde (FA), as a typical indoor chemical pollutant, has been known to be associated with development of allergic diseases, including asthma (AS) and atopic dermatitis (AD). Indeed, FA exposure occurs in every modern home and might be related to recent increases in AD (5–10).

Questions addressed

Although there have been several studies on the effects of FA exposure on immune responses, the role of FA exposure in AD is still controversial, and with regard to skin, there is little understanding of the association between FA-inhaled exposure and provocation or exacerbation of AD. In the present study, we investigated whether (i) FA provokes AD in a sensitive mouse and (ii) FA exacerbates AD provoked by house dust mite in sensitive mouse.

Experimental design

For extensive Materials and Methods, see Supplementary materials (Data S1).

Mice were divided into three groups: (i) fresh air-exposed normal control inhalation group, (ii) low (0.2 ppm $\pm$ 10%) and (iii) high concentration (1.0 ppm $\pm$ 10%) of FA-exposed group. Each group was separated into two groups and then one group was topically treated with HDM once a week, another was not. Four weeks after the last FA exposure, ear skin, blood and spleen taken from each group of mice were used for mRNA, protein and histological analysis.

Results

FA inhalation aggravated AD-like skin symptoms induced by HDM stimulation

Formaldehyde inhalation, regardless of exposed FA concentration, aggravated AD-like skin inflammation induced by topical HDM application (Fig. 1a–f). However, independent FA inhalation failed to provoke the significant AD-like skin symptoms in the atopic-prone NC/Nga mice. We also confirmed that combined exposure to FA and HDM could exacerbate the histological changes including epidermal hyperplasia and dermal infiltration of inflammatory cells such as eosinophils and mast cells (Figure S1).

FA inhalation increased total levels of IgE and IgG2a in plasma

Dual exposure to FA and HDM elevated HDM-induced total plasma IgE and IgG2a production (Table S1), but did not HDM-specific IgE (data not shown). Interestingly, total plasma level of IgE and IgG1 was increased in the group of independent FA inhalation, especially at low (0.2 ppm) concentration, but IgG2a was not (Table S1).

FA inhalation enhanced mRNA expression of cytokine in AD-like skin lesion

Quantitative real-time PCR (qPCR) analysis showed that combined exposure to FA and HDM upregulated HDM-induced skin mRNA expressions of Th1-, Th2- and Th17-related cytokines, such as IFN- γ , IL-4, IL-13, IL-17E/IL-25 and IL-17A, respectively. Our results showed that the combination of low concentration (0.2 ppm) of FA inhalation plus topical HDM stimulation significantly increased the cytokine mRNA expressions (Table 1).

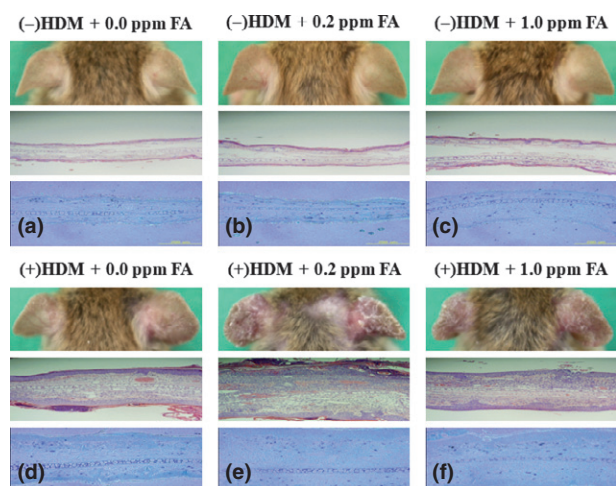


Figure 1. Effects of Formaldehyde (FA) inhalation on clinical features and histological changes in ear skin of NC/Nga mice. Photographs of the clinical features and microscopic histological images from the ear skin of NC/Nga mice in (a) fresh air-exposed normal control inhalation group, (-)HDM+0.0 ppm FA; (b) only the 0.2 ppm FA-exposed group, (-)HDM+0.2 ppm FA; (c) only the 1.0 ppm FA-exposed group, (-)HDM+1.0 ppm FA; (d) only the HDM-exposed group, (+)HDM+0.0 ppm FA; (e) dual exposure to HDM and 0.2 ppm FA, (+)HDM+0.2 ppm FA; (f) dual exposure to HDM and 1.0 ppm FA, (+)HDM+1.0 ppm FA.

Interestingly, independent FA inhalation, especially at low concentration (0.2 ppm), increased the skin mRNA expressions of IL-13 and IL-17E/IL-25 (Table 1).

We also examined whether FA inhalation affected the proallergic and diffusible Th2 promoting cytokine, TSLP, mRNA expression in skin.

As shown in Table 1, FA inhalation seemed to have an effect on the increased skin mRNA expression of TSLP and dual exposure to FA and HDM upregulated the skin mRNA expression.

FA inhalation upregulated the protein levels of Th2 cytokines in spleen

To assess the effect of FA inhalation on protein expressions of cytokines by activated T cells, splenocytes were cultured and incubated for 48 hrs in the presence of 2.5 ug/ml Con A.

As shown in Table S2, dual exposure to FA and HDM upregulated the produced protein levels of Th2- and Th17-related cytokines, such as IL-4, IL-13 and IL-17A, respectively. Interestingly, we found that topical HDM stimulation induced a slight decrease in the secreted protein level of IFN- γ in spleen, and FA inhalation downregulated the splenic IFN- γ protein produc-

tion in proportion to exposed FA concentration. We also showed that independent FA inhalation, especially at 0.2 ppm, could affect the increased IL-13 protein expressions in spleen. However, both FA inhalation and topical HDM stimulation failed to affect significant change in TSLP protein production in spleen.

FA inhalation enhanced COX-2 mRNA expression in skin

We clarified that the effect of repeated FA inhalation on COX-2 mRNA expression in the skin. As shown in Table 1, combined exposure to low concentration (0.2 ppm) of FA and HDM upregulated the COX-2 mRNA expression induced by topical HDM stimulation in the skin. Interestingly, independent FA inhalation, especially at low concentration (0.2 ppm), increased COX-2 mRNA expression in the skin.

Conclusion

Nowadays, many studies pay attention to the association between indoor environmental factors and health effects, such as an increase in the prevalence of allergic atopic diseases (1–4).

Indoor environmental pollutants possess allergy-promoting (adjuvant) effects, and the adjuvant effects of indoor chemical pollutants are influenced by exposure to micro-organisms as well as lifestyle factors, including living in closed buildings with high humidity that promotes growth of HDMs and moulds (11–14).

We speculated that consecutive exposure to air pollutant might increase the prevalence of skin irritation and AD-like skin symptoms in mice with atopic backgrounds.

In contrast to our expectation that atopic-prone NC/Nga mice might be susceptible to the air pollution, independent FA inhalation could not provoke AD-like skin inflammation in the mice. However, low concentration (0.2 ppm) of FA inhalation affected the skin mRNA expressions of IL-13, IL-17E/IL-25 and COX-2.

IL-13, which is early produced by activated T cells and mast cells for a much longer period of time than IL-4 (15), is able to sustain allergic disease and to promote IgE synthesis (16).

IL-17E/IL-25, released from Th2 cells, epithelial cells and mast cells, induces IL-4, IL-5 and IL-13 production and promotes the initiation of proallergic type 2 responses (17,18).

COX-2 was suggested to normally limit the development of Th2 cytokines and promote the development of Th1 cytokines in response to epicutaneous sensitization with antigen (19).

Based on our results, we suggested that consecutive low concentration of FA exposure might affect the imbalance of immune

Table 1. Effect of Formaldehyde (FA) inhalation on the skin mRNA expressions of cytokines and COX-2

Relative mRNA expression	IL-4	IL-5	IL-13	IL-17A	IL-17E/IL-25	TSLP	IFN- γ	COX-2
(-)HDM + 0.0 ppm FA	1.00 $\pm$ 0.33	1.00 $\pm$ 0.22	1.00 $\pm$ 0.16	1.00 $\pm$ 0.30	1.00 $\pm$ 0.20	1.00 $\pm$ 0.11	1.00 $\pm$ 0.25	1.00 $\pm$ 0.12
(-)HDM + 0.2 ppm FA	1.48 $\pm$ 0.19	1.50 $\pm$ 0.17	4.14 $\pm$ 0.37*	2.09 $\pm$ 0.19*	2.57 $\pm$ 0.35*	1.74 $\pm$ 0.69	1.38 $\pm$ 0.23	2.19 $\pm$ 0.70*
(-)HDM + 1.0 ppm FA	0.78 $\pm$ 0.22	1.16 $\pm$ 0.30	1.24 $\pm$ 0.62	0.42 $\pm$ 0.04	1.85 $\pm$ 0.52	1.89 $\pm$ 0.52	1.30 $\pm$ 0.35	0.61 $\pm$ 0.12
(+)HDM + 0.0 ppm FA	1.96 $\pm$ 0.13*	1.28 $\pm$ 0.16	1.89 $\pm$ 0.32*	17.33 $\pm$ 4.09*	1.80 $\pm$ 0.80	3.67 $\pm$ 0.39*	4.88 $\pm$ 1.31*	3.35 $\pm$ 0.75*
(+)HDM + 0.2 ppm FA	6.79 $\pm$ 0.96†	1.32 $\pm$ 0.15	9.97 $\pm$ 0.59†	55.90 $\pm$ 6.06†	4.66 $\pm$ 0.61†	6.12 $\pm$ 0.53†	11.13 $\pm$ 1.96†	7.53 $\pm$ 1.26†
(+)HDM + 1.0 ppm FA	2.07 $\pm$ 0.27	1.10 $\pm$ 0.23	1.22 $\pm$ 0.14	44.63 $\pm$ 7.35†	2.02 $\pm$ 0.05	5.64 $\pm$ 0.68†	9.91 $\pm$ 1.01†	3.94 $\pm$ 1.78

The skin mRNAs were reverse-transcribed and then cDNAs were amplified with specific primers for target genes, such as IL-4, -5, -13, -17A, -17E, TSLP, IFN- γ and COX-2, using qPCR analysis. Data are mean $\pm$ SE values (n = 5 NC/Nga mice per group).

* P < 0.05, relative to (-)HDM+0.0 ppm FA group.

† P < 0.05, relative to (+)HDM+0.0 ppm FA group.

system and IL-13, IL-17E/IL-25 as well as COX-2 might be key factors to aggravate Ag-induced AD-like symptoms.

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Author contributions

Ji-Yun Kim performed the research, designed the research study, analysed the data and wrote the paper; Mi Sook Jeong performed the research and analysed the data; Kui Young Park designed the research study and analysed

the data; Seong Jun Seo designed the research study, analysed the data and wrote the paper.

Ethics approval for animal care

All experiments with mice were performed in accordance with the regulations and the approval of the Institutional Animal Care and Use Committee of Chung-Ang University (Approval No. 10-1011) and Korea Conformity Laboratories (KCL).

Conflict of interests

All authors have no potential conflicts of interests. There are no conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplementary Materials and Methods.

Figure S1. Effects of FA inhalation on the clinical skin severity score and the infiltration of inflammatory cells in ear skin of NC/Nga mice.

Table S1. Effect of FA inhalation on the production of total levels of immunoglobulin (Ig) isotypes in plasma.

Table S2. Effect of FA inhalation on the protein expressions of cytokines in splenocytes.

Aggravation of Atopic Dermatitis-Like Symptoms by Consecutive Low Concentration of Formaldehyde Exposure in NC/Nga Mice

Supplementary Materials to Letter of the Editor

Materials and Methods (see also Experimental Design in the “Letter to the Editor”)

FA gas generation, monitoring, and analysis in an inhalation chamber

To ensure the accuracy of the FA concentration in the inhalation chambers, the chambers were evaluated two weeks prior to the beginning of the study using a model 4912 volatile solvents generator (HCT, Seoul, Korea) consisting of a liquid pump and a liquid flow-meter. The concentration in the chambers was adjusted using the flow control of the flow-meter to ensure target concentration. The FA concentration determinations in the chambers were performed as described in the National Institute for Occupational Safety and Health manual of analytical methods (NMAM; No. 2016; “FORMALDEHYDE”). The FA sampling was performed using a model 497701 Escot Elf pump personal air sampler (MSA, Pittsburgh, PA, U.S.A) connected to a Gemini twin port sampler (MSA) and a 2,4-dinitrophenylhydrazine (DNPH) cartridge. The sampler flow rate was 0.2 L/min. The FA in the cartridge was measured by high-pressure liquid chromatography (HPLC) using a model SURVEYOR apparatus (Finnigan, Las Vegas, Nevada, U.S.A.).

Animal care

Female three-week-old SPF NC/Nga mice were purchased from SLC Japan and used at 5

weeks of age (body weight, 19-21g). These mice were housed in polycarbonate cages (n = 5 per cage) in a room with controlled temperature ($23 \pm 2^{\circ}\text{C}$) and humidity ($55 \pm 7\%$) with a 12 h light/ dark cycle. The temperature and humidity ranges conformed to the OECD test guideline for inhalation exposure chambers. The mice were fed a rodent diet (Harlan Teklab; Plaster International, Seoul, Korea) and filtered water ad libitum. The body weights were evaluated at the time of grouping and once a week during inhalation allergen exposure, and the blood was collected every week during inhalation allergen exposure. All experiments with mice were performed in accordance with the regulations and the approval of the Institutional Animal Care and Use Committee of Chung-Ang University and Korea Conformity Laboratories (KCL).

Experimental conditions

Mice were divided into three groups: 1) fresh air-exposed normal control inhalation group, 2) low ($0.2 \text{ ppm} \pm 10\%$), and 3) high concentration ($1.0 \text{ ppm} \pm 10\%$) of FA-exposed group. Each group was separated into two groups: one group of NC/Nga mice was topically treated with 25 mg Df ointment (Biostir Inc., Kobe, Japan) on both ears 3 hrs after the 4% SDS treatment once a week, and the other group was treated with only 4% SDS in a total volume of 25 μl . The mice were not restrained and FA generator was not placed into nose-only inhalation units for exposure to the test atmosphere. The whole bodies of animals were exposed to fresh air and/or FA vapor through the spontaneous respiration for 6 hrs each day, five days a week for four weeks.

Measurement of skin severity scores

Severity of dermatitis was assessed macroscopically in a blinded fashion using the following

scoring procedure once a week starting from the third challenge. Total clinical skin severity scores were defined as the sum of individual scores for the following four signs and symptoms; dryness, hemorrhage (erythema), erosion (excoriation), and edema; each of these items were allocated scores of 0 to 3, where 0 = no symptoms, 1 = mild, 2 = moderate, and 3 = severe symptoms, as described previously (1).

Histological evaluation of the skin

Ear skin of mice was removed 24 hrs after the last FA exposure. The skin was fixed in 4% neutral phosphate-buffered formalin (pH 7.2) for 24 hrs, embedded in paraffin, serial sectioned at 4 μ m thickness. Each section of skin was stained with hematoxylin and eosin (H&E) to evaluate the epidermal hyperplasia and with toluidine blue (TB) to assess the mast cell infiltration. Epidermal thickness of six different randomly chosen sites was measured and number of mast cells was also determined. Digital images of the stained sections from each group of mice were microscopically analyzed with a 20 $\times$ objective, using an Olympus IX-71 microscope equipped with an Olympus DP70 digital camera and DP controller software (Olympus, Japan). Epidermal thickness (the average distance from the granular layer to the basal layer) and mast cell infiltration and degranulation were measured and quantified using an image analysis program (BMI plus software, BumMi Universe Co., Kyungki, Korea).

Blood analysis

At 29 days following initiation of exposure, mice were sacrificed, and blood was retrieved by cardiac puncture. Plasma was taken from the blood by centrifuging at 13,000 rpm for 15 min. Following plasma preparation, samples were stored at -80 $^{\circ}$ C prior to analyses.

Measurement of total IgE, IgG1, and IgG2a in plasma

The concentration of total plasma IgE, IgG1, and IgG2a was measured using mouse EIA set (BD Biosciences, San Diego, CA) and was estimated using a standard sample from the manufacturer's set. The lower limits of detection of this method for IgE, IgG1, and IgG2a were 1.6, 15.6, and 3.1 ng/ml, respectively.

Quantitation of cytokine protein levels in splenocyte supernatants

To evaluate the production of cytokines by splenocytes, spleens of an individual group were obtained, and the isolated splenocytes were resuspended in RPMI 1640 medium containing 100 U/ml penicillin, 100 ug/ml streptomycin, 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA), and 50 uM 2-mercaptoethanol (2-ME) (Sigma Chemical, St. Louis, MO). Splenocyte suspensions were seeded 3×10^6 cells per well in 24-well, flat-bottom microplates and then incubated in the absence or presence of concanavalin A (Con A) (Sigma) at 2.5 ug/ml for 48 hrs. Following the incubation, the culture supernatants were collected to determine the production levels of cytokines using a murine ELISA kit (BD and/or eBioscience).

RNA isolation and real-time quantitative PCR

Total RNA was isolated from the ear skin with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), reverse transcription was performed with RevertAidTM first strand cDNA synthesis kit (Fermentas, Hanover, MD, USA) and then cDNAs were amplified with specific primers for genes using a PCR master mix (Fermentas) according to the manufacturer's instructions. The Bio-Rad CFX96TM Real-Time PCR system was used for the relative quantification of the target genes with the SsoFastTM EvaGreen[®] Supermix. cDNAs were

amplified with primers specific for each target. The sequences of primers for PCR amplification are shown in [Table 1](#), and real-time quantitative PCR was conducted using the following conditions: 95 °C for 10 min, followed by three-step PCR with 40 cycles of denaturation at 95 °C for 10 sec, annealing at 60 °C for 10 sec, and extension at 72 °C for 10 sec. The relative expression levels of the target genes were normalized to GAPDH.

Statistical analysis

Experimental values represent the mean $\pm$ SE of five or seven mice per group. Statistical significance was determined using the Mann-Whitney and Kruskal-Wallis tests (a nonparametric test). Values of $P<0.05$ were considered significant. All analyses were conducted using SPSS version 12.0 KO software package (SPSS, Chicago, IL, USA).

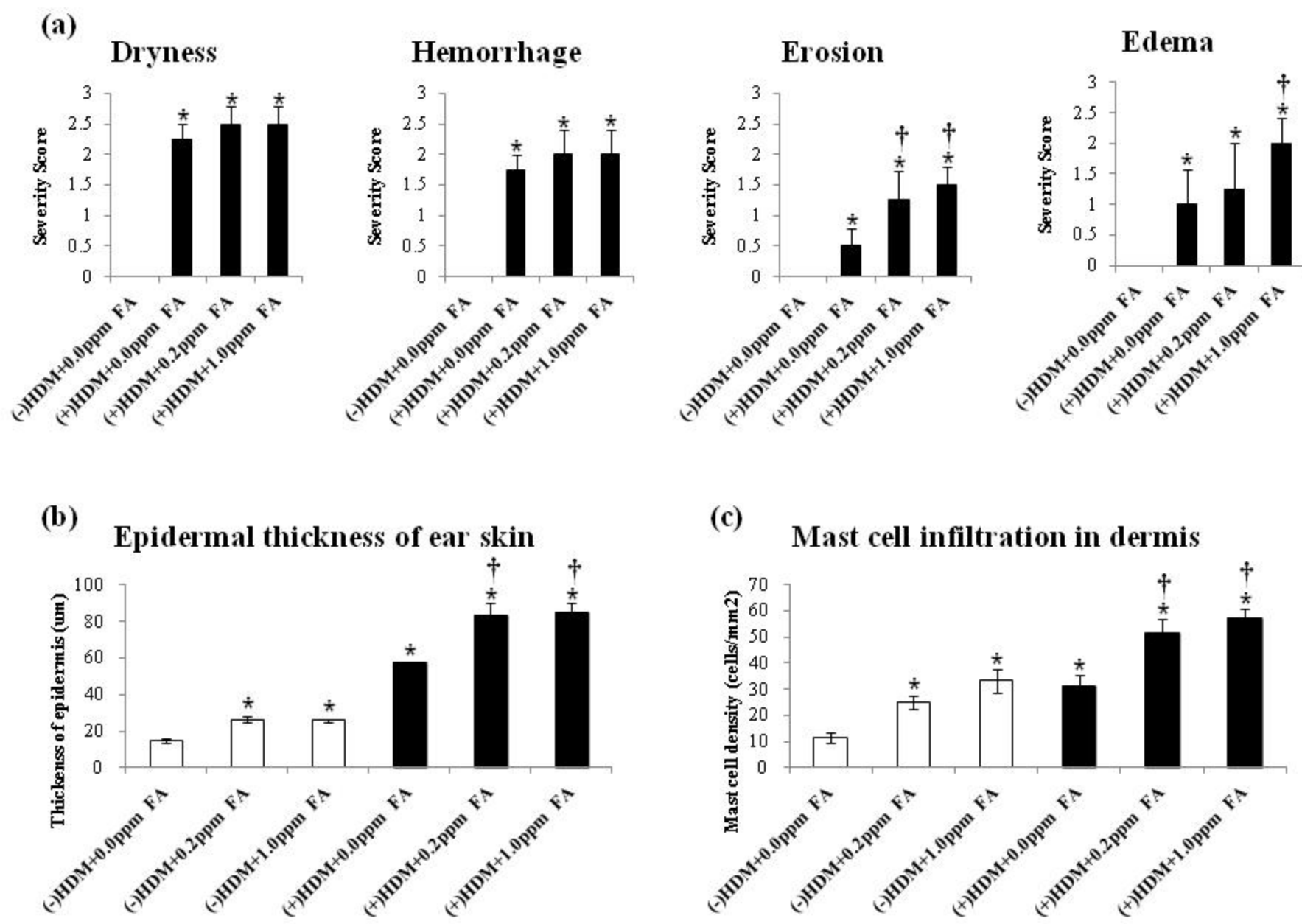
Table 1. Primer sequences

Product Name [Ⓢ]	GenBank Accession No. [Ⓢ]	Primer Sequence [Ⓢ]	Product Size (bp) [Ⓢ]
mouse IL-4 [Ⓢ]	M25892.1 [Ⓢ]	F: [Ⓢ] 5'-tcaacccccagctagttgtc-3' [Ⓢ] R: [Ⓢ] 5'-tctgtggtgttcttcgttgc-3' [Ⓢ]	455 bp [Ⓢ]
mouse IL-5 [Ⓢ]	NM_010558.1 [Ⓢ]	F: [Ⓢ] 5'-atggagattcccatgagcac-3' [Ⓢ] R: [Ⓢ] 5'-cccacggacagtgttgattct-3' [Ⓢ]	184 bp [Ⓢ]
mouse IL-13 [Ⓢ]	NM_008355.3 [Ⓢ]	F: [Ⓢ] 5'-cagcatggtatggagtgtgg-3' [Ⓢ] R: [Ⓢ] 5'-tgggtactctcgattttgtt-3' [Ⓢ]	180 bp [Ⓢ]
mouse IL-17A [Ⓢ]	NM_010552.3 [Ⓢ]	F: [Ⓢ] 5'-tccagaaggccctcagacta-3' [Ⓢ] R: [Ⓢ] 5'-agcatcttctcgaccctgaa-3' [Ⓢ]	239 bp [Ⓢ]
mouse IL-17E/IL-25 [Ⓢ]	NM_080729.3 [Ⓢ]	F: [Ⓢ] 5'-tgagttggacagggacttga-3' [Ⓢ] R: [Ⓢ] 5'-acacacacacaagccaagga-3' [Ⓢ]	237 bp [Ⓢ]
mouse IFN- γ [Ⓢ]	NM_008337.3 [Ⓢ]	F: [Ⓢ] 5'-actggcaaaaggatggtgac-3' [Ⓢ] R: [Ⓢ] 5'-tgagctcattgaatgcttgg-3' [Ⓢ]	237 bp [Ⓢ]
mouse TSLP [Ⓢ]	NM_021367.2 [Ⓢ]	F: [Ⓢ] 5'-cggatggggctaacttaca-3' [Ⓢ] R: [Ⓢ] 5'-aaatgtttgtcggggagtg-3' [Ⓢ]	216 bp [Ⓢ]
mouse COX-2 [Ⓢ]	NM_011198.3 [Ⓢ]	F: [Ⓢ] 5'-ccccacagtcaaagacact-3' [Ⓢ] R: [Ⓢ] 5'-ctcatcaccctcactcaggat-3' [Ⓢ]	191 bp [Ⓢ]
mouse GAPDH [Ⓢ]	NM_008084.2 [Ⓢ]	F: [Ⓢ] 5'-aactttggcattgtggaagg-3' [Ⓢ] R: [Ⓢ] 5'-acacattgggggttaggaaca-3' [Ⓢ]	223 bp [Ⓢ]

References

1. Suto H, Matsuda H, Mitsuishi K et al. NC/Nga mice: a mouse model for atopic dermatitis. *Int Arch Allergy Immunol.* 1999; 120 Suppl 1: 70-75.

Figure S1.



Legends for Supplementary Figures and Tables

Figure S1. Effects of FA inhalation on the clinical skin severity score and the infiltration of inflammatory cells in ear skin of NC/Nga mice.

(a) Clinical skin severity score. (b) Epidermal thickness of ear skin measured using an image analysis computer program in H&E stained sections ($\times 200$ magnification). (c) Mast cell densities in dermis measured using image analysis computer program in toluidine blue stained sections ($\times 200$ magnification). Data are mean $\pm$ SE values (n = 5 NC/Nga mice per group). * $P < 0.05$, relative to (-)HDM+0.0ppm FA group. † $P < 0.05$, relative to (+)HDM+0.0ppm FA group.

Table S1. Effect of FA inhalation on the production of total levels of immunoglobulin (Ig) isotypes in plasma.

Plasma samples were collected at 29 days following initiation of exposure. The levels of total IgE, IgG1, and IgG2a in plasma were measured by enzyme-linked immunosorbent assay (ELISA). Data are mean $\pm$ SE values (n = 5 NC/Nga mice per group). * P <0.05, relative to (-)HDM+0.0ppm FA group. † P <0.05, relative to (+)HDM+0.0ppm FA group.

Table S2. Effect of FA inhalation on the protein expressions of cytokines in splenocytes.

Spleens were isolated from each group 29 days after initiation of exposure. Splenocytes were stimulated with Con A in culture for 48 hrs. Culture supernatants were collected and the protein expressions of IL-4, -5, -13, -17A, -17E, TSLP and IFN- γ in splenocytes were measured by ELISA. Data are mean $\pm$ SE values (n = 5 NC/Nga mice per group). * P <0.05, relative to (-)HDM+0.0ppm FA group. † P <0.05, relative to (+)HDM+0.0ppm FA group.

Table S1.

Ig isotypes production	total IgE (ng/ml)	total IgG1 (ng/ml)	total IgG2a (ug/ml)
(-)HDM + 0.0ppm FA	51.41±9.30	862.74±46.06	105.94±16.49
(-)HDM + 0.2ppm FA	70.28±10.38	910.27±24.90*	109.16±22.15
(-)HDM + 1.0ppm FA	63.30±10.65	899.89±33.28	121.47±33.66
(+)HDM + 0.0ppm FA	400.72±31.60*	4210.50±85.78*	118.15±22.21
(+)HDM + 0.2ppm FA	637.75±45.91†	4417.18±60.47	159.71±24.99†
(+)HDM + 1.0ppm FA	619.00±32.46†	4476.97±78.50	98.60±16.78

Table S2.

Splenic cytokine concentration	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	IL-17A (pg/ml)	IL-17E /IL-25 (pg/ml)	TSLP (pg/ml)	IFN- γ (ng/ml)
(-)HDM + 0.0ppm FA	8.7 $\pm$ 1.2	9.9 $\pm$ 1.3	102.5 $\pm$ 24.8	35.3 $\pm$ 5.1	31.2 $\pm$ 0.4	28.5 $\pm$ 1.3	369.9 $\pm$ 38.5
(-)HDM + 0.2ppm FA	11.9 $\pm$ 1.2	35.9 $\pm$ 8.2*	261.0 $\pm$ 35.1*	46.5 $\pm$ 6.2	33.7 $\pm$ 0.8	28.9 $\pm$ 2.7	396.2 $\pm$ 18.7
(-)HDM + 1.0ppm FA	12.2 $\pm$ 1.1	31.9 $\pm$ 5.8*	196.6 $\pm$ 54.3*	46.9 $\pm$ 8.9	33.7 $\pm$ 0.9	28.8 $\pm$ 2.9	372.2 $\pm$ 30.5
(+)HDM + 0.0ppm FA	10.3 $\pm$ 0.8	11.4 $\pm$ 1.5	144.0 $\pm$ 23.1	188.9 $\pm$ 23.4*	33.0 $\pm$ 0.6	30.9 $\pm$ 1.5	315.6 $\pm$ 26.3
(+)HDM + 0.2ppm FA	19.9 $\pm$ 1.5†	12.3 $\pm$ 0.9	228.5 $\pm$ 25.5†	262.2 $\pm$ 39.5†	33.2 $\pm$ 1.2	35.3 $\pm$ 2.1	215.6 $\pm$ 53.7†
(+)HDM + 1.0ppm FA	12.5 $\pm$ 1.4	21.7 $\pm$ 3.5†	218.0 $\pm$ 28.5†	268.0 $\pm$ 38.2†	33.6 $\pm$ 1.3	32.3 $\pm$ 1.6	110.1 $\pm$ 31.0†