

Original article

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Running title: The relationship between 12 HETEs and atopic dermatitis

Quantitative determination of 12 hydroxyeicosatetraenoic acids by chiral liquid chromatography tandem mass spectrometry in murine atopic dermatitis model

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2

Atopic dermatitis (AD), one of the most important skin diseases, is characterized by both skin barrier impairment and immunological abnormalities. Although several studies have demonstrated significant relationship between atopic dermatitis and immunological abnormalities, the role of HETE in atopic dermatitis remains unknown. To develop chiral methods to characterize 12-HETE enantiomers in 1-chloro-2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis mouse model and to evaluate the effects of 12-HETE on atopic dermatitis. BALB/c mice were applied with either DNCB or acetone/olive oil (AOO) to induce atopic dermatitis. 12(R)- and 12(S)-HETEs in plasma, skin, spleen, lymph nodes were quantified by chiral liquid chromatography-tandem mass spectrometry. 12(R)- and 12(S)-HETEs in biological samples of DNCB-induced atopic dermatitis mice also increased significantly compared with AOO group, reflecting the involvement of 12(R)- and 12(S)-HETEs in atopic dermatitis. 12(R)- and 12(S)-HETEs could be useful guide to understand the pathogenesis of atopic dermatitis.

Keywords: atopic dermatitis, arachidonic acid, chiral liquid chromatography tandem mass spectrometry, ($\pm$)12-Hydroxyeicosatetraenoic acid

1 Introduction

2 Eicosanoids are signaling molecules produced by the oxygenation of arachidonic acid that are
3 synthesized by all cutaneous cell types and are involved in inflammatory responses associated
4 with allergy, injury, and acute or chronic skin disease [12]. Over 100 different eicosanoids
5 have been identified with potent bioactive signaling capacity. Several classes of eicosanoids
6 exist, including prostaglandins, prostacyclins, thromboxanes, leukotrienes,
7 epoxyeicosatrienoic acids, hydroxyeicosatetraenoic acids (HETE),
8 hydroperoxyeicosatetraenoic acids (HpETEs), and lipoxins [6].

9 Recent studies have indicated that 12 lipoxygenases (Lox), which are enantiomer enzymes
10 catalyzing the conversion of arachidonic acid to 12-HpETEs, the precursors to 12-HETEs,
11 play a crucial role in skin barrier dysfunction and induce skin diseases [5, 19]. Abstracting
12 hydrogen from C-10 of arachidonic acid, which contains reduction of Fe^{3+} to Fe^{2+} at the
13 enzyme active site, is the first reaction in the 12 Lox pathway. This reaction results in the
14 formation of an arachidonic acid radical, which reacts with oxygen and makes 12-HpETE
15 generation. Then, 12-HETE is catalyzed by glutathione peroxidase (GPx), and glutathione
16 (GSH) is oxidized to glutathione disulfide (Fig. 1A) [18].

17 The lipoxygenase family has been identified by molecular cloning (Table 1). Mammalian
18 lipoxygenase cDNAs that generate 12(R) HETE have been cloned but the cDNA have yet to
19 be investigated in detail, other than their capacity to generate 12-HETE and their localization
20 in differentiating keratinocytes [10].

21 The first report of 12-HETEs in human skin came in 1975 [7] in which the involved areas of
22 epidermis in psoriasis showed markedly increased concentrations of free arachidonic acid and
23 12-HETEs. Many chiral polyunsaturated hydroxyl fatty acids are generated by enzymatic
24 metabolism of arachidonic, linoleic, and 11, 14-eicosadienoic acid which have been identified
25 in inflamed skin scales. It contains 9-HODE, 13-HODE, 5-HETE, 8-HETE, 9-HETE, 11-

HETE, 12-HETE, 15-HETE and 15 hydroxyeicosadienoic acid (15-HEDE). From among these derivatives, 12-HETE is the one of the most abundant [1].

Atopic dermatitis (AD) is a chronic inflammatory, relapsing, non-contagious, and pruritic skin disorder. It is a major skin disease worldwide occurring at rates of 20% in children and can persist into adulthood [3, 17]. AD is caused by both skin barrier dysfunction and immunological abnormalities. It is frequently associated with elevated serum levels of IgE antibody and a T-cell (Th2)-dominant immune response [15].

We have hypothesized that a significant relationship exists between AD and ($\pm$) 12-HETEs, and the levels of ($\pm$) 12-HETEs are changed in inflammatory biological samples. In this study, therefore, we focused on the analysis of ($\pm$) 12-HETEs levels in biological samples of 1-chloro-2, 4-dinitrochlorobenzene (DNCB)-induced AD murine model by chiral liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the finding of novel and useful AD biomarker.

Materials and Methods

Chemical and Reagents

DNCB (99% purity) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The mouse IgE enzyme-linked immunosorbent assay (ELISA) kit was purchased from Shibayagi (Gunma, Japan). LC standards of 12(R)-HETE, 12(S)-HETE, ($\pm$) 12-HETEs, 12(S)-HETE- d_8 were purchased from Cayman Chemical (Ann Arbor, MI, USA). All LC standards were stored at -20°C. The high performance liquid chromatography grade glacial acetic acid and all other chemicals were purchased from J.T Baker (Phillipsburg, NJ, USA).

DNCB-induced AD

Eight week-old female BALB/c mice were obtained from Koatech (Seoul, Korea). The mice were allowed to acclimatize for 5 days before they were used in experiments. The animals

were housed in groups of five in cages identified by a card indicating the test number, animal number, test article, and duration. Room temperature (22°C), humidity (50%), and photoperiod (12 h light/12 h dark cycle) were monitored daily. The animals were allowed free access to food and water throughout the experiments. The animals were applied with 100 μ L 1% DNCB in acetone/olive oil (AOO) onto the shaved back skin at 1, 4, and 7 weeks to induce AD. Control animals were applied with AOO alone at the same times [8]. Body weight of DNCB or AOO-applied mice are measured at Day 0 and Day 49 (Table 3). All animal experimental protocols were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-120628-2).

Measurement of total IgE in serum

Serum samples were obtained by centrifugation (1700 $\times$ g, 10 min) from blood collected on day 49 after three DNCB and AOO applications and stored at -70°C until analysis. Total IgE concentration was measured using a mouse IgE ELISA kit (Shibayagi), according to the manufacturer's instructions. The complete kit was stored at 2–8°C before use. All kit reagents were brought to room temperature (20–25°C) before use.

Histopathology

At the termination of the *in vivo* study period, all animals were euthanized and examined. Skin, spleen, and lymph nodes were fixed in 10% neutral buffered formalin after necropsy, embedded in paraffin, and sectioned into 4 μ m slices. Pathological changes were evaluated and compared between groups. Selected sections were stained with hematoxylin and eosin (H&E) and toluidine blue for mast cell infiltration.

Sample preparation for LC-MS/MS analysis

Sections of mouse skin (~1.5 cm $\times$ 1.5 cm, ~0.7 g), spleen, and lymph nodes were immersed in liquid nitrogen for 1 second and then homogenized. All lipids were extracted using the Bligh and Dyer method [2]. Samples containing 1 mL NaCl were homogenized in a mixture

of chloroform: methanol (1:2, v/v) and vortexed for 10–15 min. Then, 1.25 mL chloroform was added with mixing for 1 min, and 1.25 mL NaCl was added with mixing for an additional 1 min before centrifugation. The upper phase was discarded, and the lower phase was collected through a 0.45 μ m syringe filter (13 mm cellulose acetate; Advantec, Milpitas, CA, USA). The extract was collected and evaporated in a vacuum rotary evaporator.

The residue was reconstituted in 1000 μ L of mobile phase, sonicated, and stored at -20°C until LC-MS/MS analysis. The sample was transferred to an autosampler vial, and 30 μ L was injected. The internal standard (I.S.) [12(S)-HETE-d₈] was prepared in 400 ng/mL methanol and added to all composite standards and the sample final concentration is 100 ng/mL. The peak-area ratios of compound to 12(S)-HETE-d₈ were calculated and plotted against the concentrations of the calibration standards.

Apparatus and Chiral analysis of HETEs

Chiral separation was performed on an Agilent 6430 triple quad LC/MS system with electrospray ionization (ESI) and negative ion detection (Agilent Technologies, Palo Alto, CA, USA). The ($\pm$)12-HETEs were separated using a ChiralPak@ AD-RH column (150 $\times$ 4.6 mm, 5 μ m particle size, Daicel, Illkirch Cedex, France) and isocratic elution with methanol: water: acetic acid (95:5:0.1, v/v). The system flow rate was 300 μ L/min, and the column was maintained at 40°C. The samples were maintained at 10°C prior to injection. The ESI voltage was set to 4000 V, the source temperature was 350°C, and nitrogen was the nebulizing and sheath gas. Following ionization, deprotonated molecular ions ([M-H]⁻) were fragmented by collision-induced dissociation using nitrogen gas and detected by multiple reaction monitoring (MRM) of fragment ions. The MRM transitions are summarized in Table 2. Quantification was performed using the internal and external standard method. Chromatograms for standards were used to calculate characteristic retention times of each compound, and the calibration lines were applied to verify that the MS signal was linear for

all analytical components over this range. The each analytes peak-area ratios to the I.S. [12(S)-HETE-d₈] were calculated and plotted against the concentration of the calibration standards. Calibration lines were established by the least squares linear regression method [4]. Both (R)- and (S)-enantiomers of 12-HETE were calculated relative to the corresponding 12(S)- HETE-d₈.

Statistical analysis

Data are presented as mean $\pm$ standard deviation. Statistical analyses were performed with Student's t-test for the experiments with two groups. A p-value < 0.05 was considered significant.

Results

Development of AD-like skin lesions

Skin dryness appeared initially after the repeated application of DNCB to the dorsal shaved skin of BALB/c mice, followed by mild erythema. On day 49, the skin exhibited severe erythema, scaling and hemorrhagic crust (Fig. 1B).

Histopathological changes in DNCB-induced AD mice model

A histopathological examination was performed with H&E staining of the involved skin, spleen, and lymph nodes. DNCB-treated skin revealed significant histopathological changes such as infiltration of numerous eosinophils, mononuclear cells and hyperkeratosis. Elevated mast cell numbers with mild degranulation were found under toluidine blue staining (Fig. 1C).

We also found histopathological changes in the spleen and lymph nodes of the DNCB-induced AD mice model. White pulp in the spleen of the DNCB-applied mice was heterogeneous, and the number of reticular and giant cells had increased, compared to those in AOO-applied mice. Mild lymphocyte depletion and granular lymphocytes were found in the

lymph nodes of the DNCB-applied mice (Fig 1C).

HETE enantiomers in DNCB-induced AD mice

The chiral LC-MS/MS method achieved excellent resolution of the enantiomers of each HETE regioisomer, with typical resolution of ≥ 15 min between the (R)- and (S)- forms. The (R) enantiomer eluted at ~ 10 min, the (S) enantiomer eluted at ~ 13 min, and 12(S)-HETE- d_8 eluted at ~ 13 min (Fig. 2).

The MS/MS spectra of ($\pm$)12-HETE and 12(S)-HETE- d_8 are shown in Fig. 3. The MS/MS spectra of ($\pm$)12-HETE (m/z 319 $\rightarrow$ full scan) showed two strong signals at m/z 257 and m/z 179, suggesting that the m/z 257 signal in the spectrum of ($\pm$)12-HETE was formed by loss of water (m/z 319 $\rightarrow$ m/z 301) and CO_2 (m/z 301 $\rightarrow$ m/z 257). The signal at m/z 179 was possibly due to cleavage between C-11 and C-12. Analysis of the fragmentation of the m/z 179 ion yielded a base-peak at m/z 135 (179-44).

The corresponding ions of the 12(S)-HETE- d_8 MS/MS spectrum (m/z 327 $\rightarrow$ full scan) were apparently present at m/z 264 and m/z 184 (Fig. 3). The daughter ion was formed by loss of water (m/z 327 $\rightarrow$ m/z 309) and CO_2 (m/z 309 $\rightarrow$ m/z 264).

The homogenized tissues and mouse plasma homogenates were spiked with 12(S)-HETE- d_8 to determine the linearity of extraction and the analyses of eicosanoids of interest.

Eight concentrations (range, 1–5000 ng/mL) were used to create the standard curve for calibration. The R^2 value was > 0.999 .

A representative LC-MS/MS spectrum and MRM analysis for the ($\pm$)12-HETEs in plasma, skin, spleen, and lymph nodes is shown in Fig. 4. These eicosanoids were identified with their authentic standards. The biological samples (plasma, skin, spleen, and lymph node) of DNCB-applied mice had significantly increased levels of both 12(R)- and 12(S)-HETEs compared to those in AOO-applied mice, reflecting the involvement of ($\pm$)12-HETEs in AD (Fig 5A, B). Detail averages and changes of 12(R)- and 12(S)-HETEs values of AOO- or

DNCB-applied mice (plasma, skin, spleen and lymph node) are displayed in Table 4. The values of LOD (limit of detection), LOQ (limit of quantitation), accuracy and precision in the analysis of LC-MS/MS are displayed in Table 5.

Measurement of serum IgE levels

Serum IgE levels in the AOO and DNCB-applied groups were determined by ELISA. Total serum IgE levels increased significantly in the DNCB-applied group compared with those in the AOO-applied group. The mean serum IgE level in the DNCB-applied group was 1,931.7 ng/mL. The mean serum IgE level in the AOO-applied group was 327.82 ng/mL (Fig. 5C).

Discussion

Repeated DNCB applications induce atopic AD-like skin lesions in BALB/c mice [8, 14]. In this study, AD was successfully induced by administering DNCB. This model shows clinical symptoms including skin dryness, mild erythema and edema. Finally skin thickness and scaling, severe erythema and hemorrhagic crust can be observed (Fig. 1B). The lesioned skin histologically showed significant thickening of the dermis and epidermis, hyperkeratosis, and parakeratosis. These symptomatic changes were relevant to an increased number of mast cells with mild degranulation and numerous eosinophils and lymphocytes infiltration in the skin lesions (Fig. 1C). Various chemical mediators and cytokines were released from activated mast cells which might lead to primary inflammation in the skin with infiltration of eosinophils [15]. In addition, IgE levels were increased significantly in DNCB-induced AD mice compared with those in the AOO-applied mice (Fig. 5C). Moreover, IgE levels were associated with clinical severity of AD in the DNCB treated mice which is similar to the report in patients with AD and hyper-production of IgE [6]. Various chemotactic mediators are produced from membrane lipids following IgE-dependent stimulation of immune response. These contain leukotriene B₄ and various hydroxyeicosatetraenoic acids including

12-HETE [16]. In atopic dermatitis, serum IgE level is significantly increased. Moreover, this increased IgE level could also induce the activation of immune response and generation of chemotactic mediators. This makes increase of IgE and 12-HETE levels in AD. However, for the detailed finding of relationship between IgE and HETEs in AD, additional independent works are needed.

(±)12-HETEs are potent pro-inflammatory chemotactic mediators generated by epidermal keratinocytes and dermal fibroblasts. (±)12-HETE is the predominant eicosanoid significantly up-regulated in skin diseases such as AD and psoriasis [12]. AD and psoriasis are characterized by inflammation, proliferation of keratinocyte and as mast cell accumulation and activation [13]. As the spleen and lymph nodes play an important role regulating the immune system and contain a range of immune cells, we investigated the levels of (±)12-HETEs in plasma, skin, spleen, and lymph nodes of DNCB-induced AD mice by chiral LC-MS/MS. We used MRM transitions to identify individual compounds, which were confirmed by literature sources [9]. The levels of (±)12-HETEs in homogenized samples of DNCB-induced AD were significantly elevated in plasma, skin, spleen, and lymph nodes compared to those in the control group. These results show that both 12(R)-HETE and 12(S)-HETE are associated with AD pathogenesis in mice. In addition, in a previous study demonstrated that the release of (±)12-HETE from platelets of AD patients was significantly higher compared with that in a control group [11].

Recent studies have shown that 12(R)-HETE is the up-regulated eicosanoid in psoriasis [1]. It is involved with 12(R)-lipoxygenase, which is associated with epidermal barrier function [5]. Our (±)12-HETE results are interesting because not only 12(R)-HETE increased, but also 12(S)-HETE increased significantly in biological samples (plasma, skin, spleen, and lymph nodes) of mice with DNCB-induced AD. These results indicate that 12(S)-HETE could be another key factor in AD pathology. In conclusion, levels of both 12(R)- and 12(S)-HETE in

plasma, skin, spleen, and lymph nodes from DNCB-induced atopic dermatitis mouse model increased significantly as evaluated by chiral LC-MS/MS. These results suggest that activation of the ($\pm$)12-lipoxygenase pathway may play a role in AD pathogenesis.

Acknowledgments

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Conflict of Interest

There is no conflict of interest.

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1 **Table 1.** Murine lipoxygenases

Lipoxygenase		Gene Name
1.	5-lipoxygenase (5-LO, 5-LOX)	Alox5
2.	8- lipoxygenase (8-LO, 8-LOX)	Alox8
3.	“Platelet-type” 12- lipoxygenase (P-12LO, 12-LOX)	Alox12
4.	“Leukocyte-type” 12- lipoxygenase (L-12LO, 12/15LO, 12/15-LOX)	Alox15
5.	“epidermal-type” 12- lipoxygenase (e-12LO, e-LOX-1)	Aloxe
6.	12(R)- lipoxygenase (12(R)-LO, e-LOX-2)	Alox12b
7.	Epidermis-type lipoxygenase-3 (e-LOX-3)	Aloxe3

2

3 **Table 2.** Multiple reaction monitoring (MRM) transitions for chiral liquid chromatography-
4 tandem mass spectrometry analysis of 12(R)- and (S) hydroxyicosatetraenoic acid (HETE)
5 enantiomers

Compound	[M-H] ⁻ (m/z)	MRM transition	Collision energy (eV)	Fragmentor (V)	Dwell
(±)12-HETEs	319	319 → 257	15	90	200
		319 → 179			
12(S)-HETE- d ₈	327	327 → 264	15	90	200
		327 → 184			

6

Table 3. Body weight (g) of DNCB and AOO-applied mice

	DNCB Day 0	Day 49	AOO Day 0	Day 49
Mouse 1	17.02	22.11	18.75	24.11
Mouse 2	17.71	22.80	17.91	23.01
Mouse 3	18.02	23.30	18.82	22.88
Mouse 4	17.58	22.88	18.36	22.13
Mouse 5	17.41	24.18	17.97	23.13

Table 4. Comparison of 12(R)- and 12(S)- hydroxyeicosatetraenoic acid (HETE) average levels in acetone olive oil (AOO)-applied and 1-chloro-2, 4-dinitrochlorobenzene (DNCB)-applied mice

	AOO	DNCB	Change
Plasma	1.28	4.56	About 3.56 times increase
Skin	10.62	109.46	About 10.31 times increase
Spleen	1.42	18.11	About 12.84 times increase
Lymph node	1.03	29.91	About 29.04 times increase

● 12(R) HETE (ng/mL) average of 5 samples for each of the groups

	AOO	DNCB	Change
Plasma	178.27	404.94	About 2.27 times increase
Skin	722.49	2120.66	About 2.94 times increase
Spleen	3464.20	18110.81	About 5.23 times increase
Lymph node	11.74	1350.79	About 115.06 times increase

● 12(S) HETE (ng/mL) average of 5 samples for each of the groups

Table 5. Limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision in the analysis of LC-MS/MS

Compound	LOD* (ng/mL)	LOQ** (ng/mL)	Concentration (ng/mL)	Accuracy*** (recovery %)	Precision*** (%)
12(R)-HETE	0.0078	0.0237	10	91.7	5.9
			25	100.5	2.7
			50	101.6	3.1
12(S)-HETE	0.0084	0.0253	10	90.5	6.4
			25	100.2	2.8
			50	111.2	3.7

*Limit of detection (LOD) : $3.3 \sigma/S$ (σ = standard deviation of the response. S = slope of the calibration curve). **Limit of quantitation (LOQ) : $10 \sigma/S$. ***Accuracy (recovery %), precision : average % of target value, % relative standard deviation (RSD) n=7 injections.

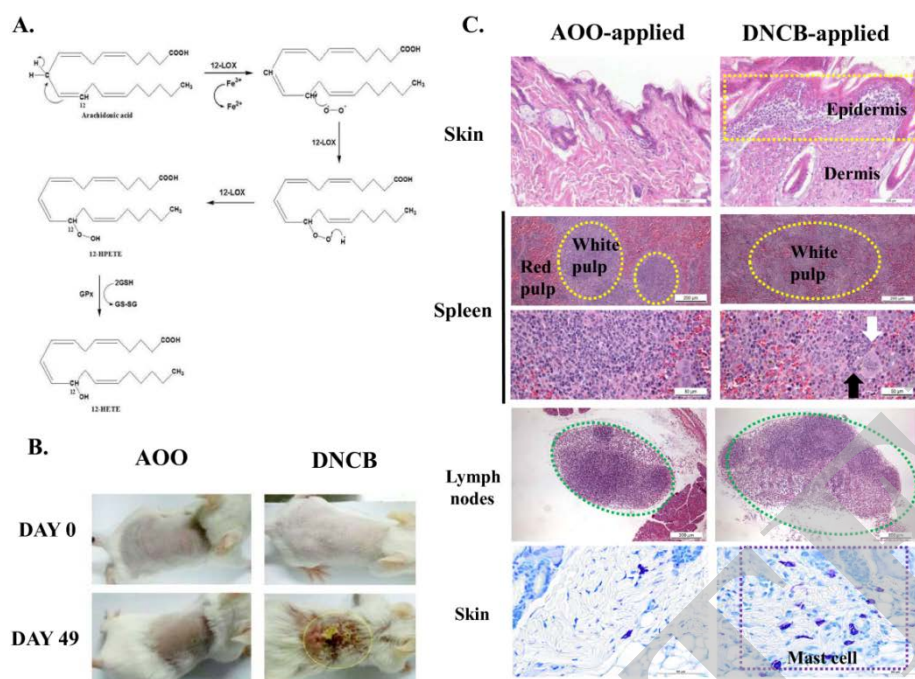
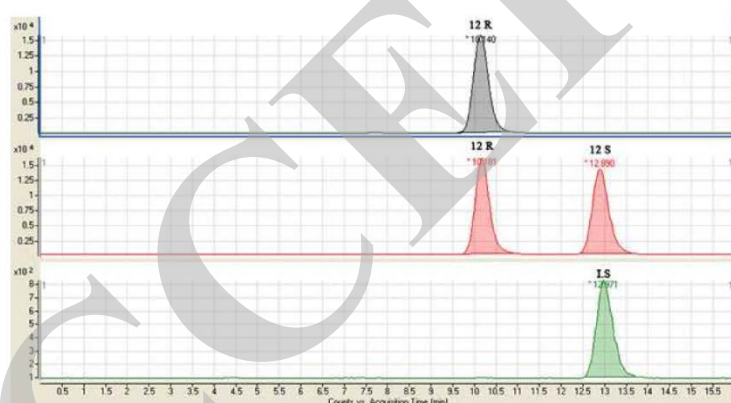


Fig. 1. (A) Mechanism of 12 lipoxygenase (Lox) catalyzed arachidonic acid transformation and comparison of (B) skin manifestations and (C) histopathology in acetone/olive oil (AOO) or 1-chloro-2, 4-dinitrochlorobenzene (DNCB)-applied mice. (A) Hydrogen is abstracted from the bisallylic methylene. Re-arrangement of the radicals causes the formation of a cis-trans conjugated diene system. The molecular dioxygen is inserted stereospecifically forming a peroxy-radical. This substance is subsequently reduced to a hydroperoxide anion. 12-HETE is catalyzed by glutathione peroxidase (GPx), and glutathione (GSH) is oxidized to glutathione disulfide [17]. (B) The upper row photos were taken before treatment with AOO and DNCB. No skin lesions were clinically detected in AOO-applied mice at 49 days. Erythema, scaling, and hemorrhagic crust (yellow circle) were observed in DNCB-applied mice at 49 days. These animals were treated with 100 μ L of 1% DNCB in AOO onto the shaved back skin at 1, 4, and 7 weeks to induce atopic dermatitis. (C) Hematoxylin and eosin (H&E) staining of dorsal skin lesions, spleen, lymph nodes and toluidine blue staining of skin lesions in AOO or DNCB-applied mice. H&E staining of DNCB-applied dorsal skin lesion revealed

1 hyperkeratosis, superficial pyoderma, and infiltration of inflammatory cells (yellow box)
2 (original magnification, $\times 200$). DNCB-applied mice spleen showed heterogeneous and
3 indistinct white pulp (yellow circle) (upper row original magnification, $\times 100$). The number of
4 reticular (black arrow) and giant cells (yellow arrow) increased in DNCB-applied mice
5 compared with AOO-applied mice (lower row original magnification, $\times 400$). H&E staining
6 of lymph nodes in DNCB-applied mice showed mild lymphocyte depletion and granular
7 lymphocytes (yellow circle) compared with those in AOO-applied mice (original
8 magnification, $\times 100$). Higher magnification of subcutaneous fatty layer in the DNCB-applied
9 dorsal skin lesion showed massive infiltration of mast cells under toluidine blue staining
10 (original magnification, $\times 400$).



11
12 **Fig. 2.** Chiral separation of ($\pm$)12-hydroxyeicosatetraenoic acid (HETE) standards by using
13 liquid chromatography-tandem mass spectrometry analysis. 12 (R)-HETE standard eluted at
14 10.140 min (Top). Chiral separation of ($\pm$)12-HETE standards; 12(R)-HETE eluted at 10.181
15 min and 12(S)-HETE eluted at 12.890min (Middle). 12(S)-HETE d_8 standard eluted at 12.971
16 min (Bottom).

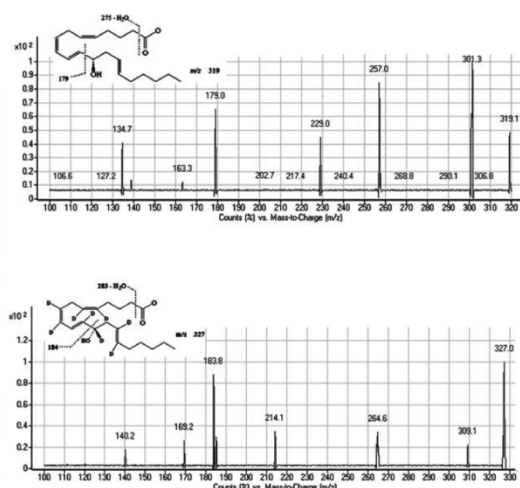


Fig. 3. Product ion mass spectra of the (A) (±)12-hydroxyeicosatetraenoic acid (HETE) standard and (B) internal standard (I.S.). Deprotonated molecules (m/z 319 for (±)12-HETE and m/z 327 for I.S.) were chosen as the precursor ions in the tandem mass spectrometry experiments.

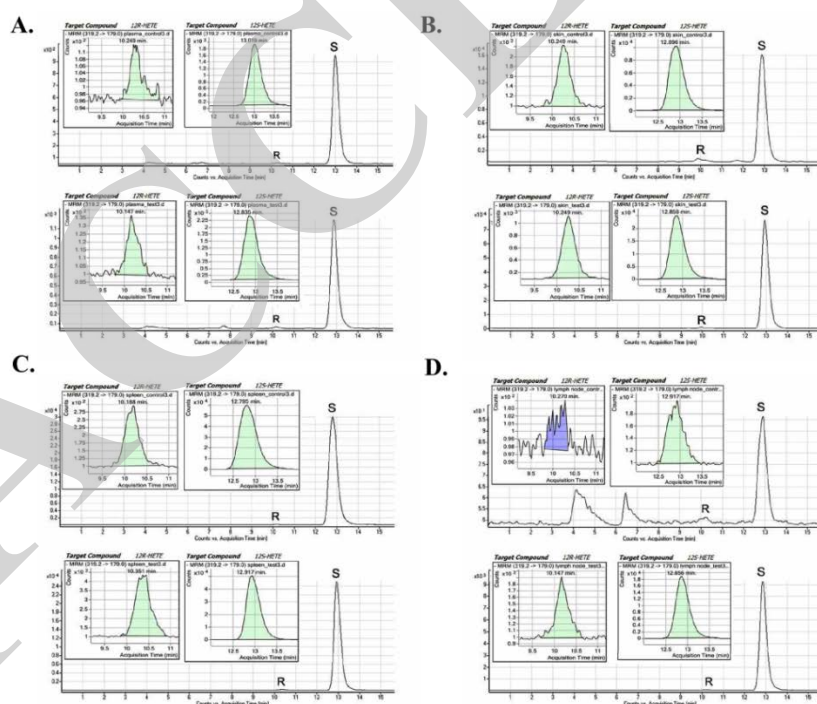


Fig. 4. Chiral LC/Ms/MS analysis and quantification of (±)12 HETE from acetone/olive oil

(AOO) or 1-chloro-2, 4-dinitrochlorobenzene (DNCB)-applied mice samples. Representative chromatograms of ($\pm$)12 HETE released from (A) plasma, (B) skin, (C) spleen, (D) lymph nodes of AOO-applied (top) and DNCB-applied mice (bottom). All ($\pm$)12 HETEs (A–D) were detected by electrospray negative ionization and multiple-reaction monitoring of the transition ions for the metabolites.

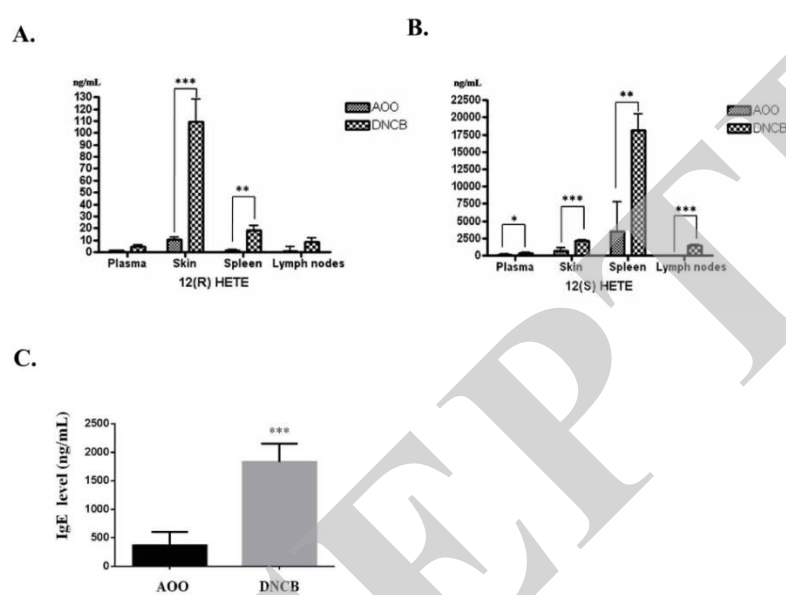


Fig. 5. (A, B) Comparison of levels of ($\pm$)12-hydroxyeicosatetraenoic acid (HETE) and (C) consecutive changes in total serum IgE levels (ng/mL) in acetone olive oil (AOO)-applied and 1-chloro-2, 4-dinitrochlorobenzene (DNCB)-applied mice. (A, B) Brackets indicate significant differences between means (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). All results are means $\pm$ standard deviations ($n = 5$). Note the different scales for 12(R)-HETE and 12(S)-HETE. (C) The mean serum IgE level of AOO-applied mice remained at a low level (327.82 ng/mL), whereas the mean serum IgE level of DNCB-applied mice increased to a very high level (1,931.7 ng/mL). Values are means $\pm$ standard deviations ($n = 5$). *** $P < 0.001$ by paired t-test compared to the AOO-applied group.