

Effect of Aerosolized Docosahexaenoic Acid in a Mouse Model of Atopic Asthma

Akihito Yokoyama^a Tomohito Hamazaki^b Akira Ohshita^a Nobuoki Kohno^a
Kimiko Sakai^a Guang-Dong Zhao^a Hitoshi Katayama^a Kunio Hiwada^a

^aSecond Department of Internal Medicine, Ehime University, Onsen-gun, ^bDepartment of Clinical Application, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan

Key Words

Ovalbumin · Soybean oil · Bronchoalveolar lavage · Eosinophil · Inhalation · Lungs · Docosahexaenoic acid · Bronchial hyperresponsiveness

Abstract

Background: Docosahexaenoic acid (DHA) found in fish oil is known to depress inflammation-related mediators. We investigated a novel delivery method of tridocosahexaenoyl-glycerol (DHA-TG). **Methods:** BALB/c mice (6–8 weeks old) were primed intraperitoneally with ovalbumin (OVA) and Al(OH)₃ on days 0 and 7, and with aerosolized OVA on day 7. Primed mice were challenged by repeated exposure to aerosolized OVA on days 15–17. Just before each exposure to aerosolized OVA, the mice were also exposed to an aerosol of emulsified DHA-TG or soybean oil, or saline (days 7 and 15–17). Bronchial hyperresponsiveness (BHR) to methacholine was measured, and bronchoalveolar lavage fluid was obtained 24 h after the last challenge (day 18). Lungs were histologically examined. **Results:** Bronchoalveolar lavage fluid of saline-treated mice showed an increased cellularity with predominant eosinophils. Exposure to DHA-TG significantly reduced the total cell number and the eosin-

ophil percentage in lavage fluid, whereas soybean oil did not. **Conclusion:** DHA but not soybean oil exposure reduced BHR and cell infiltration to bronchovascular bundles. This type of DHA administration could be studied in clinical trials.

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Introduction

Bronchial asthma is caused by a chronic inflammatory process involving a cascade of events, including a complex cell-to-cell interaction via cytokines and other inflammatory mediators [1, 2]. The incidence of asthma in Greenland Inuits, who have a high intake of marine oil, is extremely low [3], and this suggests that marine oils may have a protective effect in asthma. The highly unsaturated n-3 fatty acids (n-3 HUFA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are predominant in fatty fish. Ingestion of these fatty acids competitively replaces arachidonic acid (AA) in cell membranes. EPA is the precursor for the 3 series of prostaglandins and thromboxanes (TXs) and for the 5 series of leukotrienes (LTs). Generally, n-3 eicosanoids are much less potent than those of n-6 [4], except for cysteine-containing leukotrienes; LTC₅

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Correspondence to: Dr. Tomohito Hamazaki
Department of Clinical Application, Institute of Natural Medicine
Toyama Medical and Pharmaceutical University
Toyama-shi, Toyama 930-0194 (Japan)
Tel. +81 76 434 7615, Fax +81 76 434 5057, E-Mail hamazaki@ms.toyama-mpu.ac.jp

is equipotent with LTC₄ [5]. Dietary supplementation with n-3 HUFA can also suppress the synthesis of cytokines such as IL-1 α , IL-1 β and TNF- α from mononuclear cells [6]. Moreover, DHA can inhibit the transcription of inducible NO synthase, as described recently [7]. Since endogenous NO is reported to promote airway inflammation rather than act as a bronchodilator [8], the inhibition of NO synthesis may contribute to the suppression of inflammation in asthmatic airways. Several lines of evidence described above imply that fish oil potentially has protective effects against bronchial asthma.

A number of clinical trials investigating supplementation with fish oil in patients with asthma have demonstrated no improvement in symptoms or pulmonary function [9–12]. In contrast, the low incidence of asthma in Eskimos suggests that dietary supplementation of fish oil may reduce the risk of allergic sensitization [13]. In fact, Hodge et al. [14] recently reported that after adjusting for confounders, children who ate fresh oily fish had a significantly reduced risk of asthma, at the odds ratio of 0.26. There is a report demonstrating that dietary supplementation with fish oil (3.2 g of EPA and 2.2 g of DHA) for 10 weeks attenuated the late asthmatic response to allergen challenge compared with placebo [15]. However, this treatment did not improve any other symptoms [15]. The late response is characterized by eosinophilic inflammation in the airway [16]. Consequently, supplementation with fish oil may modulate eosinophilic airway inflammation.

We have developed infusible emulsions of n-3 HUFA to achieve a more rapid and efficient supplementation of cell membranes with n-3 fatty acids than dietary supplementation [17, 18]. Intravenous infusion of n-3 fatty acids reduces natural killer cell activity in humans [19]. Also, infusion of these fatty acids reduces LTB₄ production by neutrophils in 6 h [18, 20]. In this study, we investigated the effect of aerosolized DHA emulsion on pulmonary allergic inflammation using the recently described murine model of allergic asthma characterized by eosinophilic infiltration [21].

Materials and Methods

Mice and Experimental Protocol

Mice (BALB/c, 6- to 8-week-old females; Charles River Laboratories, Shizuoka, Japan) were primed intraperitoneally with 10 μ g of ovalbumin (OVA; grade V; Sigma Chemical Co., St. Louis, Mo., USA) and 20 mg of Al(OH)₃ in 0.2 ml of saline on days 0 and 7. On day 7, after being individually placed in a 50-ml plastic tube, the mice were also exposed to aerosolized OVA dissolved in saline

(50 mg/ml, 5 ml) or saline alone (negative control group), delivered by a DeVilbiss 646 nebulizer (DeVilbiss Corp., Somerset, Pa., USA) driven by compressed air at 5 l/min for 20 min. The primed mice were challenged by repeated exposure to OVA (50 mg/ml, 5 ml, for 20 min) on days 15–17. Exposure to an aerosol of 5 ml of emulsified DHA (DHA group), soybean oil (soybean group) or saline (positive control group) was similarly performed just prior to each exposure to aerosolized OVA (days 7 and 15–17). Two mice were exposed to aerosol at a time.

Preparation of Emulsions

Tridocosahexaenoyl-glycerol (DHA-TG) was a generous gift from Harima Chemicals Inc., Tsukuba, Ibaraki, Japan. Soybean oil was purchased from Yoshihara Oil Mill Ltd., Osaka, Japan. Those oils were emulsified at Nihon Pharmaceutical Co. Ltd., Izumisano, Osaka, Japan, according to the method of Geyer et al. [22]. One hundred milliliters of DHA-TG emulsion contained 10 g of DHA-TG (>95% pure) with 0.2% α -tocopherol, 1.2 g of egg yolk lecithin and 2.5% glycerol solution with 1 mM ascorbic acid. The composition of soybean oil emulsion was similar to that of DHA-TG emulsion, except that DHA-TG was replaced with soybean oil containing 0.2% α -tocopherol.

Bronchoalveolar Lavage Fluid and Microscopic Examination

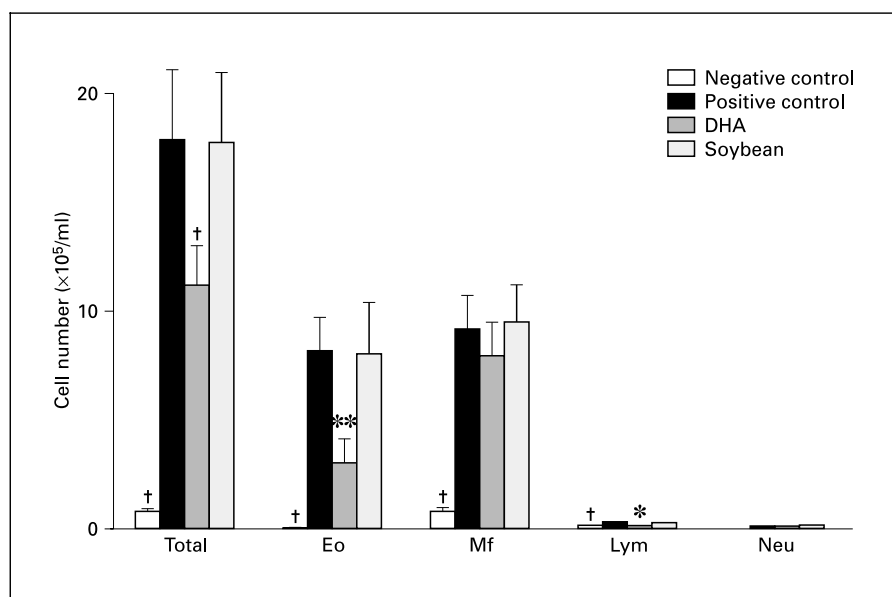
Mice were sacrificed by cervical dislocation under ether anesthesia 24 h after the last challenge (day 18). Bronchoalveolar lavage fluids (0.8 ml, 6 times) of whole lungs were obtained by cannulating the surgically exposed trachea. Cells in bronchoalveolar lavage fluid were counted using a hemocytometer. Cells were differentially counted with cytopsin preparations (May-Giemsa staining). For histological examination, the lungs were removed and fixed in 4% paraformaldehyde. Then tissues were embedded in resin (Jung HistoResin Plus, Reichert/Jung, Heidelberg, Germany) and cut into 2- μ m sections. The sections were stained with hematoxylin and eosin and assessed subjectively.

Measurements of Specific Antibody Levels and Bronchial Hyperresponsiveness

OVA-specific IgE and IgG2a antibody titers were determined by indirect ELISA, as described previously [23]. As an internal standard, pooled serum from OVA-immunized BALB/c mice was included in each assay. The standard serum was assigned values for OVA-specific IgE and IgG2a of 250 and 1,900 U/ml, respectively.

Bronchial hyperresponsiveness (BHR) was measured just before sacrifice (day 18) under conscious and unrestrained conditions by whole-body plethysmography (Buxco, Troy, N.Y., USA), as described previously [23]. In short, mice were placed individually in a whole-body chamber, and pressure differences between the main chamber containing the mouse and a reference chamber were recorded. Measurements were obtained before and after exposure to aerosolized methacholine (0, 25 and 50 mg/ml). Data were averaged for 3 min and expressed as the 'enhanced pause' (Penh): $\text{Penh} = [(\text{Te} - \text{Tr})/\text{Tr}] \times (\text{PEP}/\text{PIP})$; where Te is the expiratory time (s), Tr is the relaxation time (time for the pressure to decrease to 36% of the total box pressure during expiration), PEP is the peak expiratory pressure (ml/s), and PIP is the peak inspiratory pressure (ml/s).

Fig. 1. Total and differential cell numbers in bronchoalveolar lavage fluids. The numbers of total cells, eosinophils (Eo) and lymphocytes (Lym) were significantly decreased by treatment with aerosolized DHA. * $p < 0.05$, ** $p < 0.01$, † $p < 0.001$ versus both positive control and soybean groups. Mf = macrophages; Neu = neutrophils.



Analysis of Fatty Acid Composition

Following bronchoalveolar lavage, lungs were removed and snap frozen in liquid nitrogen, and sent to Sagami Chemical Research Center. The fatty acid composition of the total phospholipid fraction of lungs was determined as described previously [24].

Statistical Analysis

Data are expressed as the means $\pm$ SD. The variables were analyzed by ANOVA followed by an unpaired t test with Bonferroni's adjustment. A level of $p < 0.05$ was accepted as statistically significant.

Results

As shown in figure 1, exposure to DHA-TG emulsion significantly reduced both total cell number ($p < 0.001$) and the percentage of eosinophils in the bronchoalveolar lavage fluid ($p < 0.01$) compared with the positive control and soybean groups. Exposure to soybean oil did not cause any significant changes as compared with the positive control group (fig. 1).

A marked cell infiltration, predominantly to the bronchovascular bundle in the lung, was noted in positive control mice (fig. 2A). Such cell infiltration was obviously decreased by exposure to DHA-TG, but not by soybean oil (fig. 2B, C, respectively). There were no adverse effects due to exposure to aerosolized oil emulsions.

Figure 3 shows the Penh values of the mice following exposure to aerosolized methacholine. Mice of the negative control group showed mild BHR to methacholine.

The positive control mice developed greater antigen-induced BHR. Those of the soybean group showed a comparable degree of BHR to positive controls. BHR in the DHA group was reduced to the same level as the negative control mice, and was significantly less than that of the positive control and soybean oil groups.

Table 1 shows the changes in the fatty acid composition of the total phospholipid fraction of the lungs. It revealed a significant incorporation of DHA into the lung. We also calculated the availability index of AA, the ratio of AA to the sum of HUFA [25]. Because HUFA other than AA probably compete with AA, this index may parallel the degree of inflammation [25]. The index was significantly lower in the DHA group than in the other groups.

There was a sizable amount of OVA-specific IgE but not IgG2a antibodies, and there were no significant differences among the positive control, DHA and soybean groups (236 ± 124 , 207 ± 108 and 194 ± 105 U/ml, respectively).

Discussion

The present study demonstrated that an aerosolized DHA emulsion could be effective for the treatment of a murine model of bronchial asthma without undesirable adverse effects such as toxicity to the epithelium and lipid pneumonia.

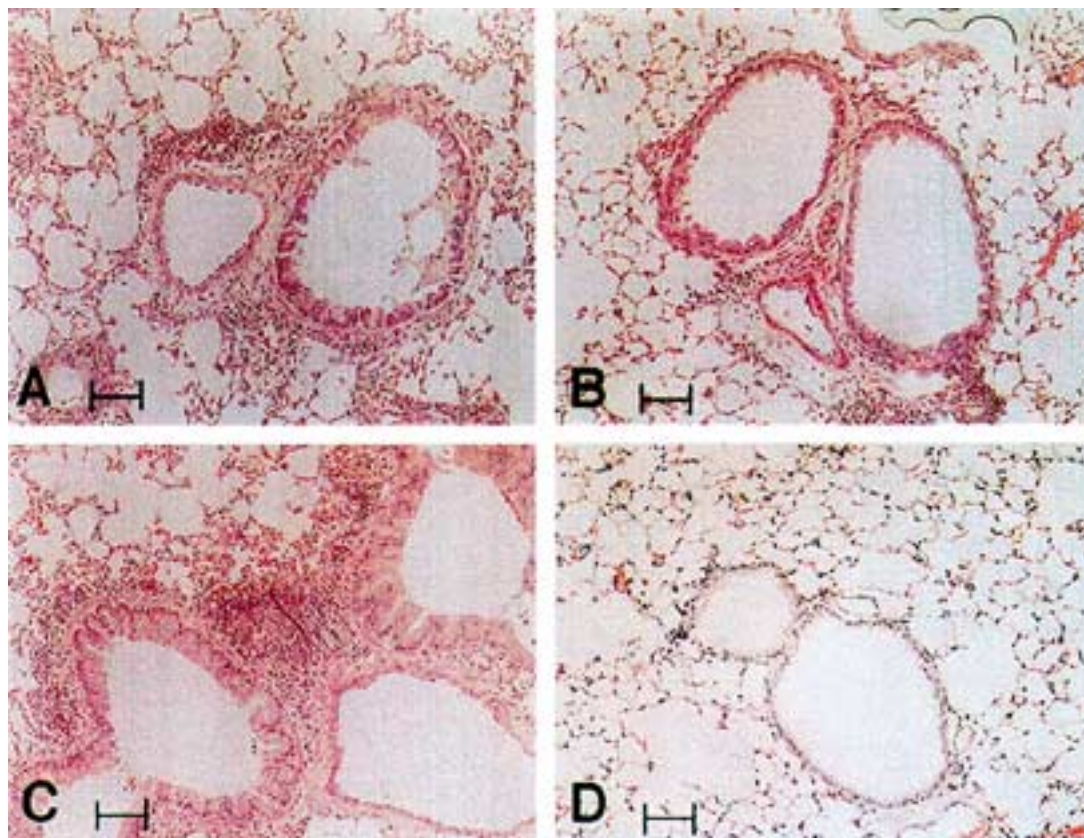


Fig. 2. Representative photographs of 2- μ m sections of the lung. **A** The positive control group. **B** The DHA group. **C** The soybean oil group. **D** The negative control group. The sections were stained with hematoxylin and eosin (original magnification $\times 100$). Cell infiltration to DHA-TG-treated lungs (**B**) is less severe compared with the positive control and soybean groups. Internal scale markers represent 100 μ m.

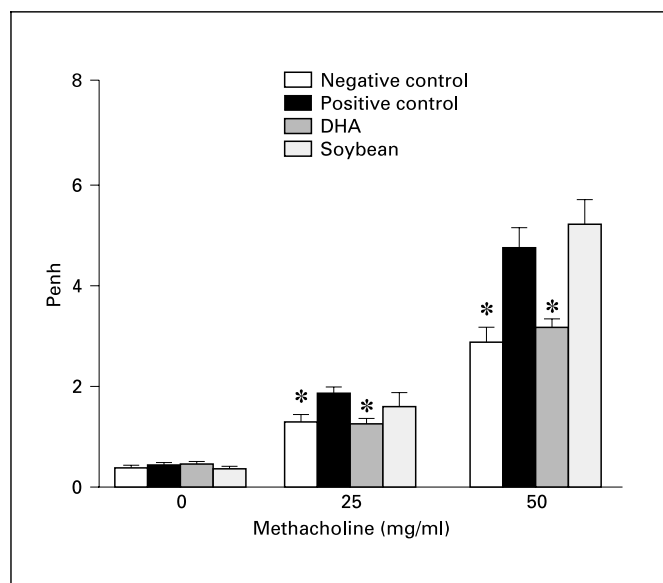


Fig. 3. BHR to methacholine. The Penh values in response to methacholine (see Materials and Methods) were significantly reduced by the treatment with aerosolized DHA. * $p < 0.01$ versus both positive control and soybean groups.

Table 1. Fatty acid composition in the total phospholipid fraction of the lung

Fatty acid	Group		
	positive control (n = 6)	DHA (n = 6)	soybean oil (n = 6)
16:0	37.3 ± 1.0	37.3 ± 1.6	36.3 ± 1.5
16:1	3.0 ± 0.2	2.8 ± 0.2	3.0 ± 0.5
18:0	12.6 ± 0.3	12.6 ± 0.7	12.9 ± 0.6
18:1n-9	8.8 ± 0.1	8.6 ± 0.3	8.5 ± 0.6
18:2n-6	5.8 ± 0.3	5.7 ± 0.2	5.6 ± 0.5
20:4n-6	10.4 ± 0.5	8.5 ± 0.4*	10.4 ± 1.3
22:4n-6	1.6 ± 0.1	1.3 ± 0.1**	1.6 ± 0.1
20:5n-3	0.4 ± 0.04 [#]	0.6 ± 0.07	0.7 ± 0.3
22:5n-3	1.5 ± 0.1	1.5 ± 0.1	1.9 ± 0.2 [#]
22:6n-3	6.8 ± 0.3	9.3 ± 0.8**	7.2 ± 0.7
Availability of AA ¹	0.50 ± 0.02	0.40 ± 0.02***	0.48 ± 0.03

Mice were exposed to aerosol of saline, or that of DHA or soybean oil emulsion on days 7 and 15–17. The lung was excised on day 18 for fatty acid analysis. * $p < 0.02$, ** $p < 0.005$, *** $p < 0.001$ (significantly different from the other groups); [#] $p < 0.001$ (significantly different from the DHA group).

¹ The availability of AA is the ratio of AA to Σ (HUFA) [25].

In addition to the possible mechanisms described in the Introduction, such as a reduction in LTB_4 and NO production induced by DHA, several other lines of evidence could account for the anti-inflammatory effect of DHA observed in this study. TXA_3 derived from DHA through EPA is much less potent than TXA_2 from AA. The anti-inflammatory effect of DHA observed in the present study could be due to TXA_3 , because inhibition of TXA_2 or blockade of the TX-prostanoid receptor could result in the attenuation of eosinophilic inflammation in mice [26]. It is known that n-3 fatty acids, including DHA, can reduce prostaglandin E_2 formation both by competitively inhibiting cyclooxygenase activity [27] and AA incorporation into the sn-2 position of phospholipids. As shown in table 1, the AA availability index, which predicted well the degree of inflammation caused by carrageenan in rats [25], was significantly lower in the DHA group. Consequently, it is highly likely that the substrate for eicosanoid synthesis was reduced in the DHA group. Dietary supplementation with fish oil inhibits the function of antigen-presenting cells [28] and the expression of class II major histocompatibility antigen, which is of particular importance in immunological responses [29]. In

contrast, it should be noted that DHA treatment did not significantly reduce the level of IgE antibodies to OVA. This suggests that DHA acts to reduce inflammation but does not reduce allergic sensitization.

The fatty acid composition in the lung phospholipid fraction was analyzed 24 h after the final exposure to aerosolized DHA-TG emulsion following extensive bronchoalveolar lavage. Furthermore, DHA in emulsion was almost exclusively in the form of triglycerol instead of phospholipids. Consequently, higher levels of lung phospholipid DHA in DHA group than in the other groups were not due to DHA-containing particles themselves in the aerosol of DHA-TG emulsion.

Swanson et al. [30] fed mice a diet containing 1.5 g of EPA and 0.5 g of DHA per 100 g of diet, and analyzed the fatty acid composition of phospholipid fractions of the lung. According to their results, it took only 3 days for DHA concentrations in the phosphatidylethanolamine fraction in the lung to increase from 11 to 16%, and in the phosphatidylcholine fraction from 1.3 to 2.9%. These increments are more or less comparable with those found in our mice of the DHA group, which showed 9.3% DHA in the total phospholipid fraction, with 6.8% for the control saline group (table 1). During the first 3 days, the daily intake of DHA of the mice in the study of Swanson et al. [30] is estimated to be about 1 g of DHA/kg of body weight. In contrast, the inhaled DHA amount from aerosol in our experiment seemed very small. Five milliliters of DHA-TG emulsion (0.5 g of DHA) was aerosolized at 5 l/min for 20 min, and 2 mice inhaled the aerosol. Assuming that the mice inhaled air at 25 ml/min and that one half of the inhaled DHA was exhaled, the daily absorbed DHA through the airways is roughly estimated to be 0.05 g/kg of body weight, which is one twentieth of the estimated dose of Swanson et al. [30]. This is due to the advantage of direct administration to the target organ over systemic administration by oral feeding.

There are severe inflammatory diseases of the lungs, such as adult respiratory distress syndrome and idiopathic pulmonary fibrosis, for which current therapy is far from satisfactory. Concurrent disturbance of blood flow is noted in these lung diseases. Since DHA has rheological effects [31] in addition to anti-inflammatory effects, inhalation therapy with DHA might be beneficial for these intractable respiratory diseases. It would take a rather large dose of n-3 fatty acids to achieve a significant increase in DHA in the lung phospholipid fraction, if administered orally; it would probably take a long period, too, in human cases. DHA aerosolization may solve these problems. The present study opens a new route for DHA

administration, i.e. inhalation, for intractable inflammatory respiratory diseases. Although our experimental mice showed no obvious evidence of adverse effects such as lipid pneumonia, these should be borne in mind for clinical applications.

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