

ORIGINAL ARTICLE

Lyprinol reduces inflammation and improves lung function in a mouse model of allergic airways disease

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Summary

Background Asthma is an inflammatory airway disease that is characterized by an influx of eosinophils to the lungs, mucus hypersecretion and T helper type 2 cytokine production. Recent dietary changes, including a decreased ω -3 polyunsaturated fatty acid (PUFA) intake, may have contributed to increased asthma rates and dietary supplementation with marine oil could have clinical benefits.

Objective To assess the effects of dietary supplementation with ω -3 PUFAs on allergic inflammation and lung function using a mouse model of ovalbumin (OVA)-induced allergic airway disease (AAD).

Methods BALB/c mice received a daily supplement of either fish oil (rich in ω -3 PUFA) or lyprinol (a complex mixture of various marine lipids plus vitamin E and olive oil) before and during AAD. The effects of supplementation on AAD were assessed.

Results Lyprinol but not fish oil treatment reduced eosinophil influx into the bronchoalveolar lavage fluid, the lung tissue surrounding the airways and the blood, decreased mucus hypersecretion in the lung and reduced airway hyperresponsiveness (AHR). The effects of lyprinol were not associated with changes in serum IgG1 or IgG2a, or the release of IL-4, IL-5, IL-13 and IFN- γ .

Conclusions Lyprinol suppresses the development of allergic inflammation and AHR in AAD. The therapeutic potential of dietary supplementation with lyprinol for asthma warrants further investigation.

Keywords: asthma, eosinophil, fish oil, lyprinol, ω -3, Th2 response

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Introduction

Allergic inflammation and asthma are characterized by increased eosinophils in the blood and lung tissue that is associated with the increased production of the T helper type 2 (Th2) cytokines IL-4, IL-5 and IL-13 [1]. The accumulation of inflammatory cells including eosinophils, as well as associated cytokines in the airways of asthmatics causes detrimental effects on airway function including airway smooth muscle contraction [2], airway hyperresponsiveness (AHR) [3], mucus hypersecretion [4], epithelial shedding [5] and vascular exudation [6]. Generally, there is a correlation between the number of eosinophils in the airways and the severity of the disease [7].

In recent decades, there has been a decrease in the consumption of ω -3 polyunsaturated fatty acids (PUFA) in the Western diet, which has been associated with an increased susceptibility to allergic conditions by predisposing to a Th2 lymphocyte response [8]. Indeed, ω -3 PUFAs have anti-inflammatory effects *in vivo* by altering membrane fluidity and eicosanoid production [9]. ω -3 PUFAs have also been shown to decrease the chemotactic responsiveness of neutrophils [10] and reduce free radical production from leucocytes [11].

Studies of the possible benefits of dietary supplementation with ω -3 PUFA in asthma have yielded conflicting results. Although there is little evidence to suggest that ω -3 PUFA is beneficial in established asthma [12], several studies have reported fish consumption to be protective against the development of asthma, particularly in childhood [13–18].

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It is possible that different forms of marine oils in the diet may have varying effects on allergic responses, due to the presence of a variety of lipid mediators as well as different quantities of ω -3 PUFAs. It has been suggested that the benefits of dietary nutrients are maximal when they are consumed in combination with the other components present in whole foods, rather than in isolation [19, 20]. Purified fish oil and the combination lipid extract, lyprinol are marine extracts that have been used in supplementation trials. Fish oil is a rich source of the ω -3 long-chain molecules eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Lyprinol is a patented extract of stabilized lipids from the New Zealand green-lipped mussels (NZGLM), *Perna canaliculus*, combined with olive oil and vitamin E (α -tocopherol) [21]. The NZGLM extract contains a complex mixture of triglycerides, sterol esters, sterols, polar lipids and free fatty acids [22–24]. Daily supplementation with lyprinol in asthmatics has been reported to decrease daytime wheeze and exhaled hydrogen peroxide concentration in a randomized clinical trial [25].

The hypothesis of this study was that daily supplementation with lyprinol before and during the onset of allergic airway disease (AAD) would decrease allergic inflammation and reduce AHR to a greater extent than the single food component, fish oil.

Materials and methods

Mice

Male BALB/c mice (aged 6–8 weeks) were obtained from the Animal Services Unit, The University of Newcastle and housed under specific pathogen-free conditions. All procedures were approved by the animal ethics committee of The University of Newcastle, Australia.

Mice were fed a background diet containing 10% w/w fat (22% energy from fat), which was low in ω -3 PUFAs (<1%), vitamin E and other antioxidants, to prevent confounding of anti-inflammatory effects of supplements (Specialty Feeds, Glen Forrest, Australia; SF05-055). The fatty acid profile of the background diet was 37% SFA, 38% MUFA and 26% PUFA. This diet is similar to the composition of a typical low-fat human diet and includes sufficient levels of linoleic acid (18:2n-6) to prevent essential fatty acid deficiency. This low-fat diet provided an appropriate background, in which to observe the effects of the ω -3 PUFA supplements.

Induction of allergic airway disease

Allergic ovalbumin (OVA/OVA) groups were sensitized by an intraperitoneal injection of OVA [50 μ g, combined with 1 mg of Alhydrogel (Commonwealth Serum Laboratories, Melbourne, Australia) in 200 μ L of 0.9% sterile saline] on

day 0. Control groups [phosphate-buffered saline (PBS)/OVA] were sham sensitized (1 mg of Alhydrogel in 200 μ L of 0.9% sterile saline). Allergic and control groups were challenged by an intranasal instillation of OVA (10 μ g in 0.9% saline) on days 12–15. AAD was assessed 24 h after the final OVA challenge [26]. Naïve mice had no intervention.

Dietary supplementation with fish oil or lyprinol

Treatment groups received a daily dose of either 200 μ L of fish oil (Blackmores, Warriewood, NSW, Australia) or 200 μ L of LyprinolTM (Blackmores), commencing 14 days before OVA sensitization. Lyprinol is a mixture of 1:2 mussel lipids: olive oil and 25 different fatty acids, which have been described previously [27]. The ω -3 PUFA content of lyprinol is EPA 5.0%, DHA 3.9%, and other ω -3 PUFA 1.9%, yielding a total ω -3 PUFA content of 10.8% [27]. Thus, 200 μ L of lyprinol/day provided an equivalent human dose of approximately 76 mg ω -3 PUFA/day. The ω -3 PUFA content of the fish oil is EPA 18% and DHA 12%, yielding a total ω -3 PUFA content of 30%. Thus, 200 μ L of fish oil/day provided an equivalent human dose of 210 mg ω -3 PUFA/day. These doses are conservative and similar to intakes that may be typically consumed as part of a Western diet [27]. The study duration of 14 days was chosen, as it has been demonstrated previously that in mouse models of inflammatory disease, including arthritis and inflammatory bowel disease, changes in inflammatory indices occur within a 3–13 day period [28–30]. Mice were anaesthetized with isoflurane, a 21-gauge ball-ended gavage needle was inserted into the stomach and nutrients were injected. Mice were held upright for 5 s after the procedure to prevent aspiration. Sham-treated OVA/OVA control groups received 200 μ L of water by gavage and PBS/OVA and naïve and controls received no treatment.

Measurement of body mass

Mice were weighed before and after experimental protocols.

Collection and analysis of inflammatory cells in blood and bronchoalveolar lavage fluid

Blood was collected by cardiac puncture, and smears from a single drop were stained with May–Grunwald Giemsa (Fronine, Taren Point, NSW, Australia). The remaining blood was centrifuged (10 000 g for 10 min) and serum was stored at -80°C until analysis [31].

Bronchoalveolar lavage fluid (BALF) was obtained by cannulation of the trachea and washing the airways lumina [2 $\times$ 0.9 mL of Hank's balanced salt solution (HBSS)]. Approximately, 0.7 mL of the instilled fluid was recovered per wash. The BALF obtained from one animal

was pooled and centrifuged (150 g at 4 °C for 5 min). Recovered cells were treated with an erythrocyte lysis buffer and resuspended in HBSS (100 µL, Thermo Scientific, South Logan, UT, USA). Cell numbers were determined using a standard haemocytometer and cells were cytocentrifuged (15 g for 3 min) and then stained with May–Grunwald Giemsa.

Differential leucocyte populations in blood and BALF were enumerated according to morphological criteria, with a minimum of 200 cells counted per slide. Slides were examined in a blinded fashion [26].

Assessment of eosinophils and mucus-secreting cells in lung tissue

Whole lungs were fixed in 10% phosphate-buffered formalin (Sigma-Aldrich, St Louis, MO, USA), sectioned and stained with haematoxylin and eosin (Poly Scientific, BayShore, NY, USA) for the identification of eosinophils, or Alcian blue/periodic acid-Schiff (Poly Scientific) for the enumeration of mucus-secreting cells (MSCs). The mean number of eosinophils or MSC per high-powered field ($\times 100$) within 100 µm of basement membrane was determined following an examination of at least 10 fields. Slides were examined in a blinded fashion [26].

Lymph node cell culture and cytokine production

Peribronchial lymph nodes (PBLN) were dispersed through 70 µm nylon mesh, treated with an erythrocyte lysis buffer (200 µL; 155 mM NH₄Cl, 12 mM NaHCO₃, 0.1 mM EDTA, pH 7.35, 5 min) and cultured (37 °C in 5% CO₂) in 96-well plates at 1×10^6 cells/well in an animal cell culture medium (0.1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES, 100 U/mL penicillin/streptomycin, 50 µM 2-ME and 10% fetal bovine serum in RPMI 1640, Thermo Scientific) with 200 µg/mL of OVA. Unstimulated wells contained cells and medium only. CD3/CD28 stimulation was used as the positive control; wells were first coated with 0.5 µg of anti-CD3 and then 0.4 µg/mL of anti-CD28 (BD Biosciences, North Ryde, Australia) was added to the medium. After 6 days, supernatants were removed and stored at –80 °C until analysis [32]. IL-4, IL-5 and IFN- γ (BD Biosciences) and IL-13 (R&D Systems, Gympie, Australia) concentrations were measured in culture supernatants by ELISA, according to the manufacturers' instructions [26].

Determination of ovalbumin-specific serum immunoglobulins

OVA-specific IgG1 and IgG2a were quantified by ELISA [33]. Plates were coated with either OVA (2 µg/well) for sample wells or unlabelled anti-IgG of corresponding isotype for standard wells. After blocking with 3% bovine

serum albumin, wells were incubated with serum or standards (mouse IgG1 or IgG2a), followed by detection with streptavidin–HRP anti-IgG1 or anti-IgG2a. Plates were developed using tetramethyl-benzidine substrate, the reactions were terminated with 0.3 M H₂SO₄ and the OD was determined at 450 nm using a Bio-Rad 680 Microplate reader (Bio-Rad, Gladesville, NSW, Australia). All reagents were from BD Biosciences.

Measurement of airway hyperresponsiveness

AHR measurements were conducted accordingly as described previously [31]. Responses to inhaled β -methacholine are expressed as the percentage change over baseline (saline control).

Statistical analysis

The significance of differences between experimental groups was analysed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison *post hoc* test for parametric data and Kruskal–Wallis with Dunn's multiple comparison *post hoc* test for non-parametric data. Lung function tests were analysed using one-way repeated-measures ANOVA. All analyses were performed using GraphPad Prism 4.03 (GraphPad Software, San Diego, CA, USA). Values are reported as mean $\pm$ SEM for parametric data and median (interquartile range) for non-parametric data, with $P < 0.05$ considered to be significant.

Results

Body mass

There were no differences between the body mass of mice between groups after the completion of experimental protocols (data not shown).

Allergic airways inflammation after dietary supplementation

The induction of AAD by sensitization and a challenge with OVA (OVA-c group) resulted in increases in the numbers of eosinophils, neutrophils and lymphocytes in the BALF compared with naïve and PBS-sensitized controls (Fig. 1). Dietary supplementation with lyprinol, but not fish oil, resulted in a decreased number of eosinophils in the BALF compared with untreated (OVA-c) controls (Fig. 1a). Neither of the marine lipid supplements resulted in a significant change in the number of neutrophils (Fig. 1b), lymphocytes (Fig. 1c), macrophages (Fig. 1d) or total cell count (Fig. 1e) in the BALF.

The development of AAD (OVA-c) also induced increases in the numbers of MSCs, eosinophils in the lung tissue surrounding the airways and eosinophils as a

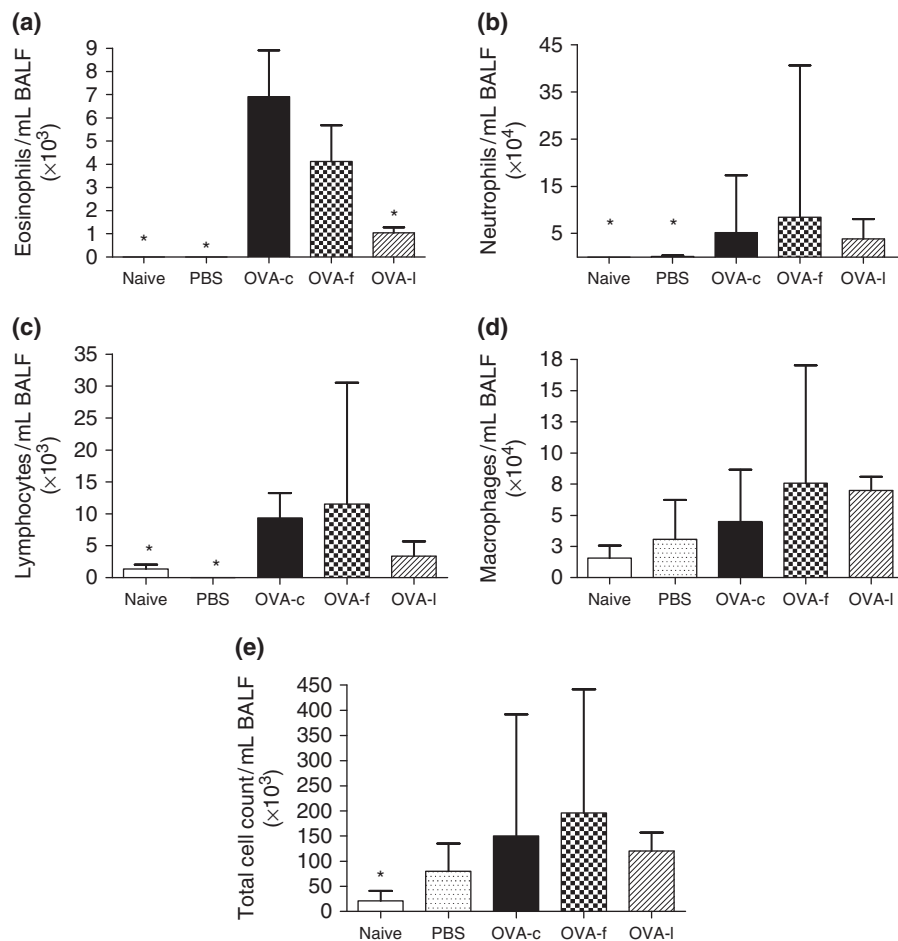


Fig. 1. Effects of fish oil or lyprinol supplementation on leucocyte levels in bronchoalveolar lavage fluid (BALF) in allergic airway disease (AAD). Experimental groups received a supplementation with either fish oil (OVA-f) or lyprinol (OVA-l) before the induction of AAD. Eosinophil influx into the airways in AAD was selectively decreased with lyprinol supplementation. (a) Eosinophils, (b) neutrophils, (c) lymphocytes, (d) macrophages and (e) total cell count are expressed as the number of cells per mL BALF. Data represent the mean $\pm$ SEM for parametric data (a), or median (interquartile range) for non-parametric data (b–e), for a minimum of six mice per group. * $P < 0.05$ compared with the OVA/OVA control group (OVA-c) as indicated. OVA, ovalbumin.

percentage of leucocytes in the blood, compared with naïve and PBS-sensitized controls (Fig. 2). Supplementation with lyprinol, but not fish oil, suppressed mucus hypersecretion in the lung (Fig. 2a). In agreement with our observations in BALF, lyprinol, but not fish oil supplementation, also reduced eosinophil influx into the lung tissue surrounding the airways (Fig. 2b) and the blood (Fig. 2c).

Effect of dietary supplementation on T helper type 2 responses

The development of AAD (OVA-c groups) led to an increase in the release of the Th2 cytokine IL-5 and a decrease in IFN- γ release from PBLN cells in response to antigen stimulation compared with naïve and PBS/OVA controls (Fig. 3). Fish oil, but not lyprinol, supplementation led to a decrease in IL-4 release (Fig. 3a). Treatment with fish oil resulted in an increased IL-13 release (Fig. 3c).

Neither of the supplements altered the IL-5 or IFN- γ levels (Figs 3b and d). CD3/28 stimulation (positive control) resulted in strong cytokine responses in each assay (data not shown).

The development of AAD (OVA-c) resulted in an increase in OVA-specific serum IgG1 antibody levels compared with naïve and PBS/OVA controls. Neither fish oil nor lyprinol supplementation altered the OVA-specific IgG1 antibody levels (Fig. 4). OVA-specific serum IgG2a values were below the ELISA detection limit for all groups (data not shown).

Airway hyperresponsiveness after dietary supplementation

The development of AAD (OVA-c) resulted in an increased AHR in terms of increased airway resistance and decreased dynamic compliance upon a challenge with β -methacholine compared with naïve and PBS-sensitized controls (Fig. 5). Supplementation with lyprinol resulted

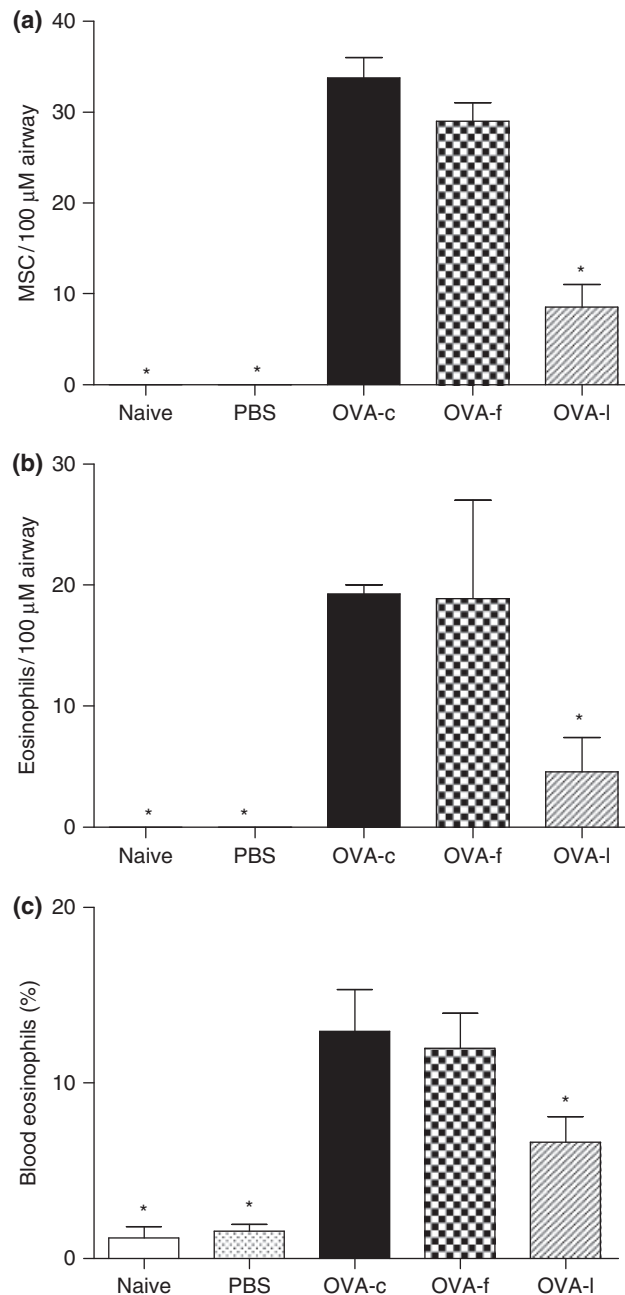


Fig. 2. Effects of fish oil (OVA-f) or lyprinol (OVA-l) supplementation on mucus-secreting cells (MSCs) and eosinophils in allergic airway disease (AAD). (a) MSC hyperplasia and eosinophil levels in (b) tissues surrounding the airways and (c) blood in AAD were decreased with lyprinol supplementation. Data represent the mean $\pm$ SEM for a minimum of three mice per group. * $P < 0.05$ compared with the OVA/OVA control group (OVA-c) as indicated. OVA, ovalbumin.

in a decreased AHR (resistance) at methacholine doses of 12.5 mg/mL and higher (Fig. 5a), but did not affect the dynamic compliance (Fig. 5b). Fish oil supplementation did not alter the AHR.

Discussion

Dietary changes over recent years, including a decreased ω -3 PUFA intake, have been suggested to have contributed

to increases in asthma prevalence [20]. This study demonstrates that dietary marine oil supplements can suppress eosinophilic inflammation and mucus production in the lungs and airways in AAD as well as Th2 cytokine responses from draining lymph nodes, which are associated with reduced AHR. The results suggest that lyprinol, but not fish oil, may provide a protective effect against asthma, as a result of its anti-inflammatory properties.

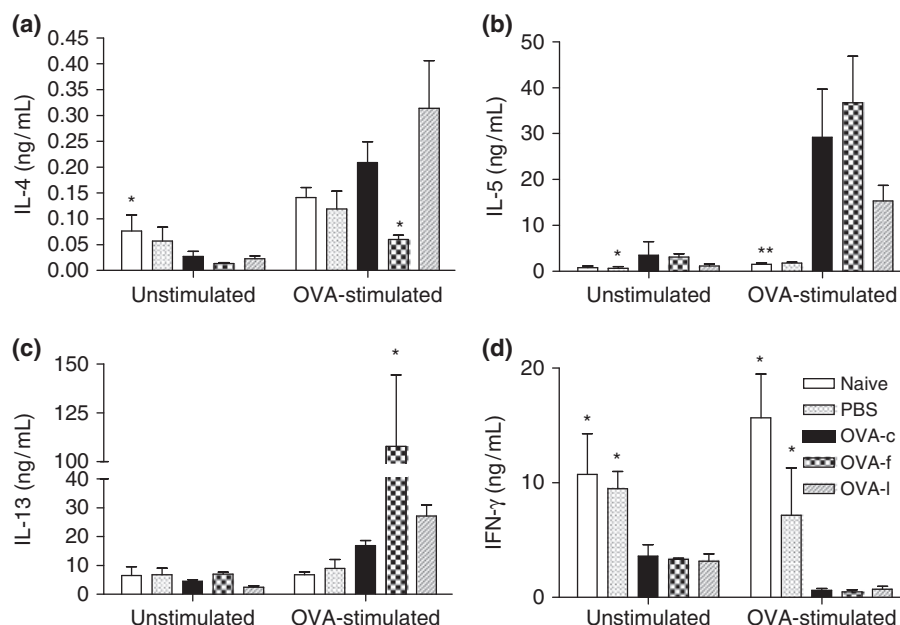


Fig. 3. Effects of fish oil (OVA-f) or lyprinol (OVA-l) supplementation on antigen-specific Th2 cytokine release in allergic airway disease (AAD). Fish oil supplementation suppressed (a) IL-4 but increased (c) IL-13 release from lymph node cells re-stimulated with antigen. (b and d) IL-5 and IFN- γ levels were not altered with either marine oil supplement. Values are expressed as ng/mL of cell culture supernatant. Data represent the mean $\pm$ SEM for a minimum of eight mice per group, with two mice pooled per data point. * P < 0.05 compared with the OVA/OVA control group (OVA-c) as indicated. OVA, ovalbumin.

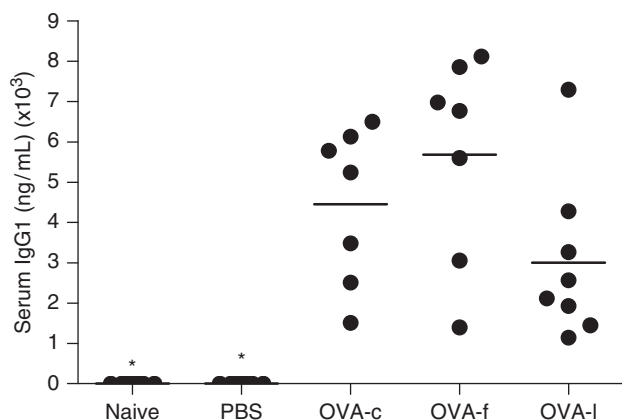


Fig. 4. Effects of fish oil (OVA-f) or lyprinol (OVA-l) supplementation on antigen-specific serum IgG1 levels in allergic airway disease (AAD). OVA-specific serum IgG1 was not altered with either marine supplement. Each data point represents results from an individual mouse with a minimum of seven mice per group. Values are expressed as ng/mL of IgG1 detected in the serum. * P < 0.05 compared with the OVA/OVA control group (OVA-c) as indicated. OVA, ovalbumin.

In this study, eosinophil numbers in the BALF, lung tissue and blood, were decreased with lyprinol, but not fish oil supplementation. Our data are in agreement with the results of a previous study, which reported that when administered at similar doses, lyprinol was more effective than fish oil at reducing inflammatory responses in a

mouse model of experimental ulcerative colitis, despite the fact that fish oil contains a higher amount of EPA and DHA [28]. The observation that fish oil supplementation did not alter inflammation or lung function, may provide an explanation for the inconsistent results of fish oil supplementation trials in asthma, several of which have demonstrated no benefit of fish oil in asthma [34–38]. It is possible that fish oil supplements are less effective than consuming whole fish, because the synergistic effects between multiple components in whole foods may be more potent than individual nutrients. Indeed, many of the studies that have reported benefits of ω -3 PUFA consumption in asthma have used a whole food approach, using fish, rather than fish oil consumption [14–17].

Studies published previously have suggested that the anti-inflammatory properties of lyprinol can be attributed to the ω -3 PUFA content, because lyprinol inhibits the 5-lipoxygenase and cyclo-oxygenase eicosanoid pathways [30, 39]. These pathways result in the production of leukotrienes (LT) such as LTC₄ and LTD₄, which enhance mucus production [40], and LTE₄, which attracts inflammatory infiltrates such as eosinophils into the airways [41]. In addition, EPA and DHA are converted to bioactive metabolites such as resolvins and protectins with anti-inflammatory and pro-resolving properties [42]. Resolvin E1 has been shown to reduce airway inflammatory cell recruitment, AHR and the production of pro-inflammatory cytokines, in a murine model of allergic airways

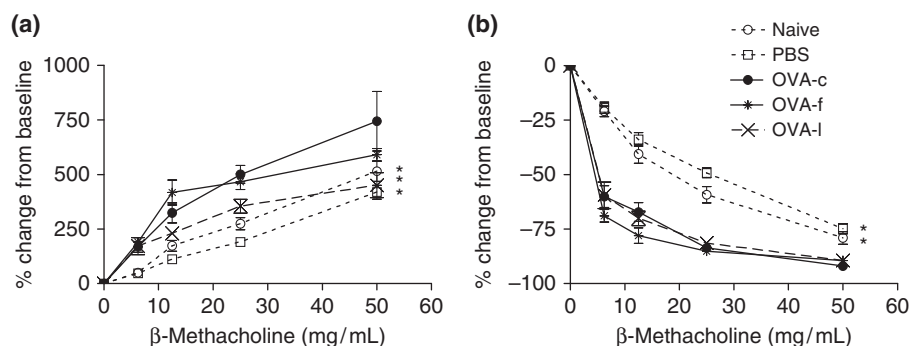


Fig. 5. Effects of fish oil (OVA-f) or lyprinol (OVA-l) supplementation on airway hyperresponsiveness (AHR) in allergic airway disease (AAD). Lyprinol, but not fish oil, supplementation suppressed lung resistance compared with untreated controls in AAD at β -methacholine doses ≥ 12.5 mg/mL. (a) Lung resistance and (b) dynamic compliance are expressed as a percentage of the baseline reactivity to saline in the absence of cholinergic stimuli. Data represent the mean $\pm$ SEM for a minimum of six mice per group. * $P < 0.05$ compared with the OVA/OVA control group (OVA-c) as indicated. OVA, ovalbumin.

disease [43]. Although these mechanisms cannot be excluded, the results of our study suggest that ω -3 PUFA alone are not responsible for the decrease in eosinophils, as fish oil, which is a richer source of ω -3 PUFA than lyprinol, did not alter the eosinophil levels. Lyprinol contains various molecules such as triglycerides, sterols, sterol esters, fatty acids, α -tocopherol and other compounds, mixed in olive oil, which is a rich source of anti-inflammatory polyphenols [28]. Our data suggest that other nutrients in lyprinol are necessary to achieve the full beneficial effects. For example, the anti-oxidant component of lyprinol may be necessary to protect cell membranes from an increased susceptibility to oxidation, which occurs due to the incorporation of long-chain PUFAs [44–46].

The reduction in BALF, lung tissue and blood eosinophils, observed with lyprinol was not associated with the suppression of several pro-inflammatory cytokines, which are often linked to eosinophilic airway inflammation. There was no difference between IL-5 levels with lyprinol supplementation compared with untreated controls with AAD. A variety of mediators, including IL-13 and GM-CSF and the eotaxins, also promote the development and chemotaxis of eosinophils, respectively [47, 48]. Our data suggest that lyprinol suppresses increases in eosinophils via such non-IL-5-dependent mechanisms. Similarly, a decreased antigen-specific IL-4 production from draining lymph nodes was observed with fish oil, but not lyprinol treatment, suggesting that the anti-inflammatory effects of lyprinol are not IL-4 dependent. The anti-inflammatory effects of lyprinol were also not associated with the modification of antigen-specific IgG1 and IgG2a levels. However, it is possible that lyprinol may down-regulate the expression of Fc γ R on lymphoid and myeloid cells, which may then attenuate the inflammatory response. The differing effects of the two supplements may also result from differential alterations and effects on tissues and other factors. However, elucidating these effects would be

a major undertaking. These potential alternate mechanisms, which may underpin the suppressive effects of lyprinol, are interesting areas for further research.

Lymph node antigen-specific IL-13 release was increased as a result of fish oil supplementation. IL-13 induces AHR in mice [49, 50]; however, the increase in IL-13 that was observed with fish oil supplementation was not related to any changes in lung function. Conversely, lyprinol did not alter the IL-13 release, but did reduce AHR. IL-13 release was measured in an *ex vivo* culture of OVA-stimulated T cells from PBLNs. Although IL-13 is not decreased, it is likely that the levels are lower in the lung. We have shown previously that a decrease in eosinophil influx can result in lower pulmonary levels of IL-13, and this may have resulted in the reduction in AHR in our study [51]. The reduction in AHR observed with lyprinol supplementation is consistent with the results of a randomized control trial. This trial investigated the beneficial effects of daily lyprinol supplementation in asthmatics and found an improvement in the morning peak expiratory flow and daytime wheeze in the group receiving supplementation [25].

In conclusion, this study provides evidence in a mouse model of AAD that supplementation with lyprinol may have beneficial effects in asthma by reducing eosinophilic infiltrates and mucus secretion in the lungs, and reducing AHR. The mechanism by which lyprinol exerts these beneficial effects remains to be elucidated, but is not associated with a reduced release of the cytokines IL-4, IL-5, IL-13 or IFN- γ in lymph nodes or serum IgG responses. It is likely that while the mechanism of action of lyprinol may involve ω -3 PUFA content and the suppression of the 5-lipoxygenase and cyclo-oxygenase eicosanoid pathways. However, the lack of efficacy of fish oil suggests that there are other nutrients in lyprinol that are essential for anti-inflammatory activity. It is likely that the efficacy of any nutritional supplement depends on the synergistic effects between the multiple nutritional

components. Thus, interventions using a whole food approach offer most promise for the future.

Acknowledgements

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