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ORIGINAL ARTICLE

# Docosahexaenoic Acid (DHA) and Docosapentaenoic Acid (DPAn-6) Algal Oils Reduce Inflammatory Mediators in Human Peripheral Mononuclear Cells In Vitro and Paw Edema In Vivo

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**Abstract** The anti-inflammatory activity associated with fish oil has been ascribed to the long-chain polyunsaturated fatty acids (LC-PUFA), predominantly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Here we examined the anti-inflammatory effects of two DHA-rich algal oils, which contain little EPA, and determined the contribution of the constituent fatty acids, particularly DHA and docosapentaenoic acid (DPAn-6). In vitro, lipopolysaccharide (LPS)-stimulated Interleukin-1 beta (IL-1$\beta$) and Tumor Necrosis Factor-alpha (TNF-$\alpha$) secretion in human peripheral blood mononuclear cells (PBMC) was inhibited with apparent relative potencies of DPAn-6 (most potent) > DHA > EPA. In addition, DPAn-6 decreased intracellular levels of cyclooxygenase-2 (COX-2) and was a potent inhibitor of pro-inflammatory prostaglandin E2 (PGE2) production. DHA/DPAn-6-rich DHA-S$^{TM}$ (DHA-S) algal oil was more effective at reducing edema in rats than DHA-rich DHA-T$^{TM}$ (DHA-T), suggesting that DPAn-6 has anti-inflammatory properties. Further in vivo analyses demonstrated that feeding DPAn-6 alone, provided as an ethyl ester, reduced paw edema to an extent approaching that of indomethacin and enhanced the anti-inflammatory activity of DHA when given in combination. Together, these results demonstrate that DPAn-6 has anti-inflammatory activity and enhances the effect of DHA in vitro and in vivo. Thus, DHA-S algal oil may have potential for use in anti-inflammatory applications.

**Keywords** Inflammation · DHA · DPAn-6 · Long-chain polyunsaturated fatty acids

**Abbreviations**

| | |
|---|---|
| ARA | Arachidonic acid |
| COX | Cyclooxygenase |
| DHA | Docosahexaenoic acid |
| DPAn-6 | Docosapentaenoic acid |
| EPA | Eicosapentaenoic acid |
| GRAS | Generally recognized as safe |
| IL-1$\beta$ | Interleukin-1 beta |
| LPS | Lipopolysaccharide |
| LC-PUFA | Long chain-polyunsaturated fatty acids |
| OLA | Oleic acid |
| PBMC | Peripheral blood mononuclear cells |
| PGE2 | Prostaglandin E2 |
| TNF-$\alpha$ | Tumor Necrosis Factor-alpha |

## Introduction

Chronic or uncontrolled inflammation can lead to tissue damage and is an underlying factor in atherosclerosis, rheumatoid arthritis, diabetes (type 1), asthma, inflammatory bowel disease, and Alzheimer's disease [1–7]. Inflammation results from the release of chemical mediators from activated leukocytes that have migrated to target areas as a consequence of tissue injury or invading pathogens. These inflammatory mediators include IL-1$\beta$,

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TNF-α, and PGE2. EPA (20:5n-3) and DHA (22:6n-3), LC-PUFA found in fish oil, have anti-inflammatory activity and affect inflammatory mediator production [8–12], lymphocyte proliferation [13, 14], monocyte and neutrophil migration [15], and natural killer (NK) cell activity in vitro [16].

The consumption of fish oil results in the replacement of arachidonic acid (ARA; 20:4n-6) in cell membranes by DHA and EPA [17, 18]. This leads to alterations in the eicosanoids produced and contributes towards a less inflammatory environment [17–20]. EPA and DHA can also be converted into resolvins, which are bioactive molecules that play an important role in the resolution of inflammation [4, 21, 22]. Further, LC-PUFA modulate the expression and activity of genes involved in the inflammatory response. In vitro, EPA or DHA reduced the production of proinflammatory cytokines, including IL-1$\beta$ and TNF-α [8–12, 20] and cell migration [23–26]. DHA and EPA may have differing mechanisms of action [27]. PBMC derived from humans supplemented with DHA had a decreased ex vivo T cell activation whereas EPA supplementation had no significant effect [28]. DHA was also more efficacious at reducing pro-inflammatory cytokines than EPA, particularly IL-1$\beta$ and IL-6 in vitro [8, 12].

While many studies to date have examined the anti-inflammatory effects of DHA and EPA from fish oil, this study focused on DHA and DPAn-6 (docosapentaenoic acid; 22:5n-6) derived from algal sources which contain little EPA. We assessed the anti-inflammatory activity of DHA/DPAn-6-rich DHA-S$^{TM}$ (DHA-S) and DHA-rich DHA- T$^{TM}$ (DHA-T) algal oils[1] and their individual constituent LC-PUFA. To our knowledge, this is the first study to show that DPAn-6 has potent anti-inflammatory activity and enhances the effects of DHA.

## Materials and Methods

### Fatty Acid Sodium Salts for In Vitro Experiments

All in vitro assays used sodium salts of EPA, ARA, DPAn-6, and Oleic acid (OLA) that were purchased from NuChek Prep (Elysian, MN) and were greater than 99% pure. DHA-sodium salt (greater than 98% pure) was purchased from Sigma-Aldrich (St. Louis, MO). Fatty acid sodium salts were prepared by resuspending in distilled water and warming for 1.5 h at 37 °C to ensure solubility before storing at −80 °C. Before use, fatty acids were again warmed to 37 °C for 10 min to ensure homogeneity. The fatty acids were used at the concentrations described in the specific in vitro assays below. Since potencies of the fatty acids were being directly compared in experiments, the concentrations of the fatty acid stock solutions were confirmed by gas-liquid chromatography, accuracy ±5%, both prior to and after the freeze/thaw process.

### Human Cell Culture

PBMC were isolated by Ficoll-Hypaque (Amersham, Uppsala, Sweden) density gradient from the peripheral blood of healthy humans per the manufacturer's instructions. Human blood obtained from healthy individuals (negative for HIV and Hepatitis-B and-C) was purchased from All Cells, Emeryville, CA. Isolated PBMC were frozen in heat inactivated fetal calf serum (FCS)/10% dimethylsulfoxide (DMSO) until use when they were thawed, placed in supplemented RPMI 1640 containing 10% FCS (heat inactivated), 1% L-glutamine (200 mM), 100 unit/ml penicillin, and 100 μg/ml streptomycin (all reagents Sigma-Aldrich, St. Louis, MO). PBMC were thawed, washed in supplemented RPMI, and cell viability was assessed by trypan blue exclusion immediately before use in vitro.

### In Vitro LPS Stimulation of PBMC

Human PBMC were stimulated with LPS (derived from *E. coli*, Sigma-Aldrich, St. Louis, MO) using a modification of the method originally described [29]. Briefly, PBMC were resuspended in supplemented RPMI 1640, seeded at $2 \times 10^5$ cells/200 μl/well of a 96-well flat-bottom plate. PBMC were pretreated for 2 h at 37 °C with 2 μl of the individual fatty acid sodium salts or an equivalent volume of water (negative control); five replicate wells per treatment. Fatty acid concentrations were used at 100 μM or 10–100 μM (dose response). PBMC were then stimulated with 20 μl LPS (0.1 ng/ml final concentration) for 18 h at 37 °C. Following stimulation, cells were pelleted by centrifugation and supernatants were collected and stored at −20 °C for cytokine measurement by ELISA. Cell viability after LPS incubation was monitored by trypan blue exclusion before and after centrifugation and no significant differences in viability were noted between control and fatty acid-treated groups at the doses used in this study.

### IL-1$\beta$ and TNF-α ELISA

Cell supernatants were tested for IL-1$\beta$ and TNF-α (Research Diagnostics, Minneapolis, MN) following the manufacturer's instructions.

[1] DHA-T oil is also known as DHASCO or DHASCO-T oil; DHA-S was formerly known as DHASCO-S oil. Both are manufactured by Martek Biosciences Corporation.

### Determination of COX-1, COX-2, and PGE2 Levels

Human PBMC were seeded at $1 \times 10^6$ cells/ml in a 24-well plate with 1 ml supplemented RPMI 1640 per well. Cells were pretreated with 10 μl of the individual fatty acid sodium salts (100 μM) or a combination of two fatty acids (50 μM each for 100 μM total) for 2 h prior to stimulation with 10 μl LPS (0.1 ng/ml); replicates of 6 wells per treatment. Cell viability after LPS incubation was monitored by trypan blue exclusion before and after centrifugation and no significant differences in viability were noted between control and fatty acid-treated groups at the doses used in this study. IL-4 (10 ng/ml (10 μl); BD Biosciences, San Jose, CA) was used as a positive control. After approximately 18 h, PGE2 levels in the supernatants were assayed using an EIA kit (Cayman Chemical, Ann Arbor, MI). For the detection of intracellular COX-1 and COX-2, PBMC were stained for 15 min with PerCP-conjugated CD14 (BD Biosciences, San Jose, CA) to identify the monocytes. After washing, cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) following the manufacturer's instructions. Cells were then stained with COX-1-FITC/COX-2-PE antibody combination or the isotype-matched controls (BD Biosciences, San Jose, CA). CD14 + gated cells, 5,000–10,000 per sample, were analyzed on a Coulter Epics Altra flow cytometer using EXPO32 Multi-COMP software (Beckman Coulter, Fullerton, CA).

### Animals and Diets

Male Sprague-Dawley rats (100–150 g) from Harlan (Indianapolis, IN) and/or Charles River Laboratories (Wilmington, MA) were quarantined for at least 3 days prior to study initiation. Food and water were provided ad libitum. AIN-76A Diet (Research Diets, New Brunswick, NJ) was used as the base diet and for all diet formulations used in the animal studies as it does not contain any LC-PUFA. Study protocols were approved by the animal facility's Institutional Animal Care and Use Committee and followed the Animal Care and Use Guidelines established by the Office for Laboratory Animal Welfare (OLAW)/NIH. Food consumption, animal body weights, and general health were monitored throughout the studies.

### DHA-S, DHA-T, ARASCO™

DHA-S, DHA-T, ARASCO oils, derived from *Schizochytrium* sp., *Crypthecodinium cohnii*, and *Mortierella alpine*, respectively, were manufactured by Martek Biosciences Corporation (Columbia, MD) and their fatty acid compositions are described in Table 1. Both DHA oils are used in food and dietary supplements [30] and are Generally Recognized as Safe (GRAS) for food. DHA-T oil in combination with ARASCO oil, an oil rich in ARA, is GRAS for the fortification of infant formula. DHA-S oil has been used in a number of clinical studies [31–33], as has the DHA-T oil [30, 34]. LC-PUFA are present as triglycerides within these oils.

**Table 1** Fatty acid profiles of oils

| | % Total fatty acids | | |
|---|---|---|---|
| | DHA-S | DHA-T | ARASCO |
| 14:0 | 8.55 | 14.69 | 0.67 |
| 16:0 | 22.79 | 12.97 | 13.74 |
| 18:0 | 0.63 | 0.69 | 8.72 |
| 18:2 (n-6) | 0.32 | 1.21 | 7.32 |
| 20:4 (n-6) ARA | 0.80 | <0.1 | 43.61 |
| 20:5 (n-3) EPA | 2.11 | 0.25 | 0.15 |
| 22:5 (n-6) DPA | 16.26 | 0 | 0 |
| 22:6 (n-3) DHA | 40.39 | 41.9 | 0 |
| Others | <8.15 | <30 | <25 |

### Evaluation of the Algal Oils in a Rodent Model of Carrageenan Paw Edema (CPE)

Rats ($n = 10$ per group) were fed modified AIN-76A rodent diets that were formulated by Research Diets (New Brunswick, NJ) to contain one or more of the following test oils: DHA-S, DHA-T, ARASCO oil and equivalent levels of total fat (5% by weight). The non-test article fat in the experimental diets and the total fat in the placebo diet consisted of a blend of 30% corn, 54% soybean, and 16% coconut oils. Oils were provided by Research Diets (New Brunswick, NJ). The fatty acid content of the formulated diets was confirmed by gas chromatography (GC). In brief, fats in the diet were transesterified in situ with 1.5 N HCl in methanol (Sigma-Aldrich, St. Louis, MO), in the presence of toluene (Sigma-Aldrich, St. Louis, MO) and an internal standard. The resultant fatty acid methyl esters (FAME) were extracted with toluene. The FAME were separated, identified, and quantitated by gas-liquid chromatography with flame ionization detection (GLC-FID) and internal standard calibration.

Experimental diets each provided 1.2% DHA, which is equivalent to approximately 800 mg DHA/kg per day. The ARASCO and DHA-S-containing diets provided approximately 300 mg of ARA or DPAn-6/kg per day (approximately 0.45% of each PUFA), respectively. After 28 days of ad libitum feeding, rats were injected with 0.1 ml of a 10-mg/ml carrageenan solution in water (Sigma-Aldrich, St. Louis MO) in the subplantar right hind foot. Paw volumes were measured by water displacement and differences between the initial and the resultant paw volumes

were determined at 3 h post-carrageenan injection. Animals were euthanized by carbon dioxide ($CO_2$) asphyxiation after final paw measurements were made.

To determine the minimum efficacious concentration in vivo, DHA-S oil was diluted with corn oil to achieve required doses ranging from 100 mg/kg per day PUFA (DHA + DPAn6 combined) to 1,500 mg/kg per day. In this experiment and those following, rats ($n = 8$ per group) were fed the AIN-76A diet for 7–10 days before administration of test compounds to allow for a wash-out of the LC-PUFA that can be found in typical rodent chows. DHA-S oil was administered by oral gavage (to ensure accuracy of dosing) daily for 14 days to allow plasma levels of LC-PUFA to equilibrate [35]. Doses less than 1,500 mg/kg PUFA per day were equilibrated to 1,500 mg/kg total fat with corn oil. Control and indomethacin treated animals received corn oil by gavage. All test materials contained equivalent levels of antioxidants: ascorbyl palmitate (Tap1010, Vitablend, The Netherlands), mixed tocopherols (Tocoblend L70 IP, Vitablend, The Netherlands), as well as rosemary extract (Herbalox, Kalamazoo, MI). Indomethacin (Sigma-Aldrich, St. Louis, MO) was administered by intraperitoneal injection (i.p.) at 5 mg/kg 30 min prior to carrageenan challenge. The hind paw assay was performed as described above with paw measurements at 2, 4, and 6 h. Immediately following final paw measurements, animals were anesthetized with isoflurane (NLS Animal Health, Owings Mills, MD), 3–5 ml of blood was collected into EDTA-treated tubes by exsanguination via cardiac puncture, and animals were euthanized. Blood was separated by centrifugation, 1,800 rpm for 20 min, and plasma was stored at −80 °C for fatty acid analyses.

### Dose-Response Evaluation of DPAn-6 in a Rodent CPE Model

To test the dose-response effect, DPAn-6 was administered at 300, 700 or 1,000 mg/kg per day and compared to DHA doses of 700 or 1,000 mg/kg as well as a DHA/DPAn-6 combo (2.5:1 ratio similar to DHA-S) at 1,000 mg/kg. This LC-PUFA mg/kg dose is equivalent to the dietary dose administered as DHA-S in the previous studies. Doses less than 1,000 mg/kg per day were equilibrated to 1,000 mg/kg total fat with a blend of control fatty acid ethyl esters that mimicked the fatty acid blend found in the base AIN-76A diet: 25% oleate, 13% palmitate, 60% linoleate, 2% alpha-linolenate. Control and indomethacin-treated animals received the control blend of ethyl esters as well. The PUFA, provided as ethyl esters (DHA, DPAn-6, OLA, palmitic acid, linoleic acid, and alpha-linolenic acid) that were used in the animal dosing experiments were purchased from NuChek Prep (Elysian, MN). These fatty acid ethyl esters were greater than 90% pure as confirmed by gas liquid chromatographic techniques. Total fat in the animal diets was 6.25% (by weight) including the 5% in the base diet and the addition of the fatty acid ethyl esters received by gavage. Indomethacin was dosed as previously described. Hind paw measurements and plasma collection were performed as previously described.

### Fatty Acid Methyl Ester (FAME) Analysis

Plasma lipids were extracted and phospholipids were isolated by thin-layer chromatography as previously described [36]. In brief, fatty acids from plasma phospholipids were saponified with sodium hydroxide and methanol and then methylated with boron trifluoride (all reagents from Sigma-Aldrich, St. Louis, MO). The resulting methyl esters were identified and quantified by gas–liquid chromatography and flame ionization detection as described [36].

### Statistical Analyses

Data were analyzed and compared by one-way ANOVA using the Bonferroni test to compare groups (GraphPad, Prism, Version 4.0). Effects were considered to be statistically significant at $p < 0.05$.

## Results

### DPAn-6 Has Potent Anti-Inflammatory Activity In Vitro

The effect of the fatty acids on IL-1$\beta$ and TNF-$\alpha$ secretion was examined. Both DHA and DPAn-6 markedly reduced IL-1$\beta$ levels in LPS-stimulated PBMC, by approximately 80 and 90% respectively ($p < 0.01$) (Fig. 1a). TNF-$\alpha$ levels were reduced approximately 80% upon treatment with either DHA or DPAn-6 (not significant) (Fig. 1b). EPA reduced the expression of these cytokines to a lesser degree, approximately 60% for IL-1$\beta$ (not significant) and 40% for TNF-$\alpha$ (not significant) (Fig. 1a, b). Overall, the relative effectiveness of the fatty acids appeared to be DPAn-6 (most potent) > DHA > EPA with respect to reducing both IL-1$\beta$ and TNF-$\alpha$. Neither OLA nor AA significantly affected IL-1$\beta$ or TNF-$\alpha$ secretion. We focused our subsequent studies on the fatty acid effects on IL-1$\beta$, as the effect was more striking.

### Effects of the Individual and Combinations of Fatty Acids on IL-1$\beta$ Secretion In Vitro

We compared the potency of the individual fatty acids in a dose response assay. Treatment of human PBMC with each of the LC-PUFA resulted in a dose-dependent reduction in

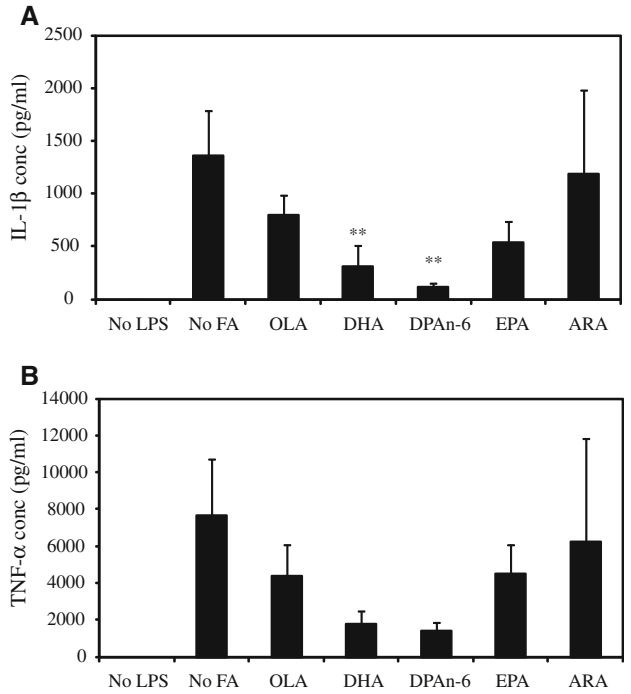


**Fig. 1** DPAn-6 has potent anti-inflammatory activity in vitro. Human PBMC were pretreated with individual fatty acids (100 μM final concentration) for 2 h prior to stimulation with LPS. Supernatants were harvested and IL-1$\beta$ (**a**) and TNF-α (**b**) levels in the culture medium were measured by ELISA following 18 h of culture. Means (5 wells/treatment) +/− SD. $^{**}p < 0.01$ compared to control. Results are representative of at least three independent experiments

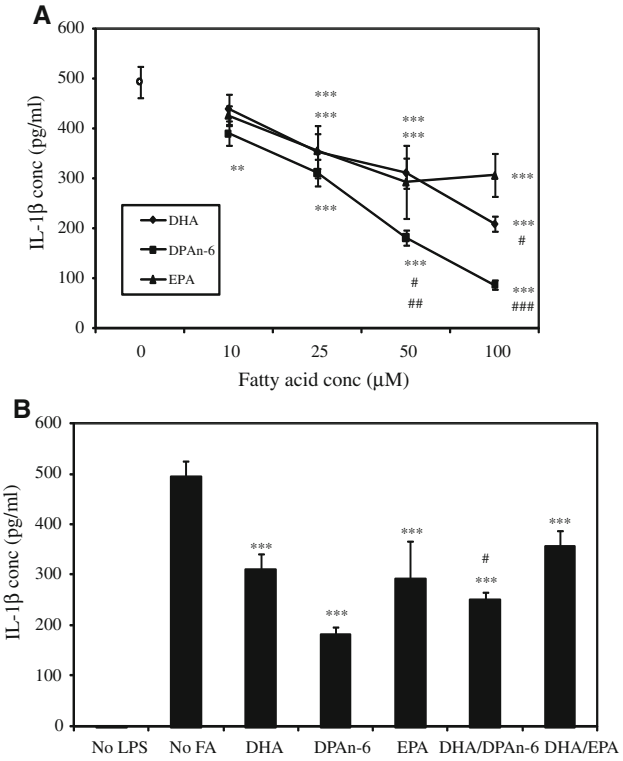


**Fig. 2** Effects of the individual and combinations of fatty acids on IL-1$\beta$ secretion in vitro. **a** Human PBMC were pretreated with the fatty acids at various concentrations 2 h prior to stimulation with LPS. IL-1$\beta$ levels in the culture supernatants were assessed by ELISA. Means (5 wells/treatment) +/− SD. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control. $^{\#}p < 0.01$ compared with an equivalent concentration of EPA. $^{\#\#}p < 0.001$ compared with an equivalent concentration of DHA. $^{\#\#\#}p < 0.001$ compared with an equivalent concentration of DHA or EPA. Results are representative of at least three independent experiments. **b** Human PBMC were incubated with 50 μM of the individual fatty acids or a combination of 25 μM of each fatty acid (50 μM total) prior to stimulation with LPS. Means (5 wells/treatment) +/− SD. $^{***}p < 0.001$ compared to control. $^{\#}p < 0.01$ compared with DHA/EPA. Results are representative of at least three independent experiments

IL-1$\beta$ (Fig. 2a). DPAn-6 significantly reduced IL-1$\beta$ with as little as 10 μM ($p < 0.01$). Overall, DPAn-6 was the most effective with maximum inhibition at 100 μM reaching approximately 80% ($p < 0.001$) versus approximately 60% for DHA ($p < 0.001$) and 40% for EPA ($p < 0.001$). Further, at a concentration of 50 μM, DPAn-6 reduced IL-1$\beta$ levels more effectively than DHA ($p < 0.001$) or EPA ($p < 0.01$). Similar results were seen with 100 μM where DPAn-6 was more effective than DHA ($p < 0.001$) or EPA ($p < 0.001$). DHA was more effective than EPA at 100 μM ($p < 0.01$).

Human PBMC were next incubated with 50 μM of the individual fatty acids or a combination of 25 μM of two fatty acids (50 μM total) prior to stimulation with LPS (Fig. 2b). In PBMC, the DHA/DPAn-6 (50 μM total LC-PUFA) combination reduced IL-1$\beta$ levels approximately 50% ($p < 0.001$), whereas the DHA/EPA combination reduced levels approximately 30% ($p < 0.001$). The DHA/DPAn-6 combination was more effective than the DHA/EPA combination in reducing IL-1$\beta$ production ($p < 0.01$).

## DPAn-6 Reduces COX-2 and PGE2 Production In Vitro

In LPS-stimulated PBMC, DPAn-6 (100 μM) inhibited levels of intracellular COX-2 approximately 50% ($p < 0.001$) (Fig. 3a). DHA did not affect COX-2 levels in these cells whereas EPA and the DHA/EPA combination increased COX-2 levels approximately 25–30% ($p < 0.01$ and $p < 0.05$, respectively). None of the fatty acids affected COX-1 levels: COX-1 mean fluorescence intensity (MFI) was 81 (control) and ranged from 81 to 90 upon treatment with the various fatty acids (data not shown). As expected, the IL-4 control decreased COX-2 levels ($p < 0.001$) without affecting COX-1 levels [37]. The DHA/DPAn6 combination was more effective than the DHA/EPA combination ($p < 0.001$). As seen in Fig. 3b, DPAn-6 also reduced the levels of PGE2 by approximately 90% ($p < 0.001$). DPAn-6 was similar in efficacy to the positive control IL-4, which is known to decrease PGE2 levels [37, 38]. Interestingly, while COX-2 levels were not affected by DHA, PGE2 levels were decreased approximately 70% ($p < 0.001$) by DHA and the

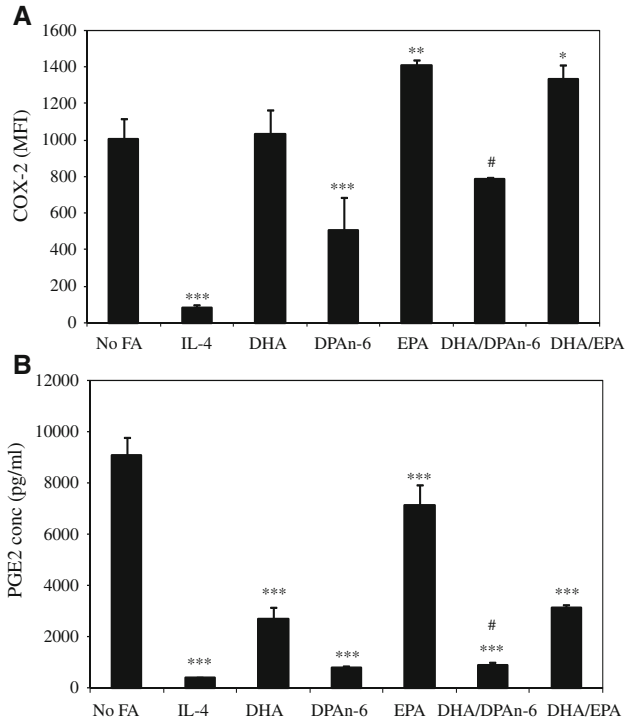


**Fig. 3** DPAn-6 reduces COX-2-mediated inflammation in vitro. **a** Intracellular levels of COX-2 in LPS-stimulated cells were determined using flow cytometry following treatment with the individual fatty acids (100 μM) and the fatty acid combinations for a total of 100 μM. Data indicate mean +/− SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the control. #$p < 0.001$ compared to DHA/EPA. **b** PGE2 levels in the culture supernatants were assessed by EIA after treatment with the individual fatty acids or combinations thereof (100 μM total). Means (6 wells/treatment) +/− SD. ***$p < 0.001$ compared to the control. #$p < 0.001$ compared to DHA/EPA. Results are representative of three independent experiments

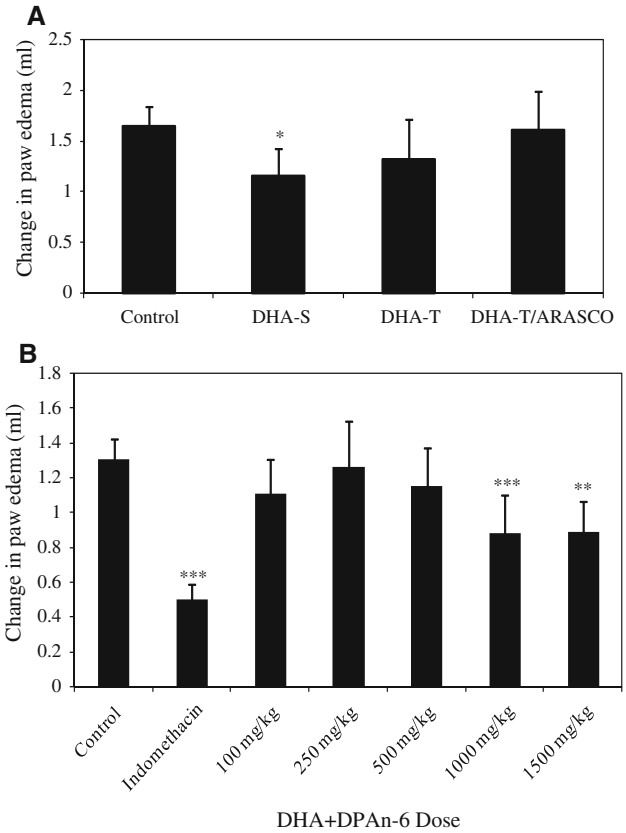


**Fig. 4** DHA/DPAn-6-rich DHA-S oil significantly reduced inflammation in a rodent paw edema model. **a** DHA-S, DHA-T, DHA-T/ARASCO oils were administered in the feed for 28 days prior to carrageenan challenge. The experimental diets provided equivalent amounts of DHA (approximately 800 mg/kg) with an additional 300 mg of DPAn-6 or ARA/kg per day for the DHA-S and ARASCO diets, respectively. Paw edema was measured by water displacement 3 h post-challenge with carrageenan stimulation. Means ($n = 10$) +/− SD. *$p < 0.05$ compared to control. **b** DHA-S oil was diluted with corn oil to achieve required doses ranging from 100 mg/kg PUFA (DHA + DPAn6 combined) to 1,500 mg/kg per day. DHA-S oil was administered by oral gavage for 14 days. Doses less than 1,500 mg/kg PUFA per day were equilibrated to 1,500 mg/kg total fat with corn oil. Control and indomethacin treated animals received corn oil by gavage. Indomethacin was administered at 5 mg/kg i.p. 30 min prior to carrageenan challenge. Paw edema was measured at 2 h post carrageenan treatment. Means ($n = 8$) +/− SD. **$p < 0.01$ and ***$p < 0.001$ compared to control

addition of DPAn-6 reduced levels further. In comparison, EPA reduced PGE2 levels approximately 20% ($p < 0.001$), and when EPA was provided in combination with DHA, PGE2 was decreased to levels comparable to those with DHA alone ($p < 0.001$). Further, the DHA/DPAn-6 combination was a more potent inhibitor of PGE2 production than the DHA/EPA combination ($p < 0.001$).

## DHA/DPAn-6-rich DHA-S Oil Significantly Reduces Inflammation in a Rodent Paw Edema Model

The anti-inflammatory activity of the DHA-rich algal oils was evaluated in a rodent model of acute inflammation. No significant differences in food intake, weight, or locomotor activity were observed between any of the treatment groups. DHA-S-fed animals had a 30% reduction in mean paw edema ($p < 0.05$), while treatment with DHA-T also tended to reduce paw edema with an approximately 20% reduction (not significant) (Fig. 4a). The animals fed the DHA-T/ARASCO diet had no reduction in paw edema.

Animals were treated with increasing doses of DHA-S oil to determine the minimum efficacious concentration in vivo. Indomethacin reduced paw edema 62% ($p < 0.001$) (Fig. 4b). Maximal inhibition of paw edema, a reduction of 33%, was visible at 1,000 mg/kg DHA + DPAn-6 ($p < 0.001$). As expected, treatment with increasing doses of DHA-S oil resulted in increases in plasma phospholipid levels of both DHA and DPAn-6 (Table 2).

### DPAn-6 Reduces Inflammatory Edema in a Dose-Response Rodent Model of Hind Paw Edema

A study was performed to assess the effects of various doses of DPAn-6 on paw edema in this model (Fig. 5). Fatty acids were provided as ethyl esters. Indomethacin reduced paw edema 62% ($p < 0.001$). The 700 mg/kg dose of DPAn-6 reduced paw edema maximally, a 56% reduction ($p < 0.001$). In addition, we confirmed our original findings that the DHA/DPAn-6 combination, as found in the DHA-S oil, reduced edema more than DHA alone (DHA-T oil) ($p < 0.05$). The 700 mg/kg DPAn-6 was as efficacious as the combination of 700 mg/kg DHA + 300 mg/kg DPAn-6. At 2 h post-challenge, treatment with 700 and 1,000 mg/kg DHA reduced paw edema approximately 20% (not significant). However, inhibition seen with 700 mg/kg DHA was increased further by the addition of 300 mg/kg DPAn-6, the PUFA combination found in the DHA-S oil. By 4 h post-challenge, both doses of DHA significantly reduced paw edema ($p < 0.01$, data not shown). DHA and DPAn-6 (1,000 mg/kg doses) elevated plasma levels approximately 4 and 6.5-fold, respectively (Table 3). Higher doses of pure DPAn-6 (700 and 1,000 mg/kg) led to increased plasma ARA levels.

## Discussion

The immunomodulatory effects of fish oil and its constituent omega-3 LC-PUFA have been examined in numerous

**Table 2** Plasma phospholipid fatty acid levels in rats treated with DHA-S oil

| Fatty acid | Treatment groups (Total PUFA dose) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | 100 mg/kg | 250 mg/kg | 500 mg/kg | 1,000 mg/kg | 1,500 mg/kg | Indomethacin |
| n-3 | | | | | | | |
| 20:5n-3 EPA | 0 (0) | 0.12 (0.03) | 0.34 (0.03)* | 0.77 (0.09)* | 1.03 (0)* | 1.56 (0.26)* | 0 (0) |
| 22:6n-3 DHA | 1.59 (0.12) | 3.42 (0.32)* | 4.52 (0.39)* | 5.1 (0.38)* | 6.73 (0.32)* | 8.13 (0.83)* | 1.56 (0.16) |
| n-6 | | | | | | | |
| 20:4n-6 ARA | 21.96 (0.55) | 19.79 (0.78)* | 20.13 (1.25)* | 18.18 (0.56)* | 19.34 (1.11)* | 19.69 (1.48)* | 20.66 (0.76) |
| 22:5n-6 DPAn6 | 1.13 (0.25) | 0.57 (0.07)* | 0.59 (0.03)* | 0.82 (0.05)* | 1.40 (0.13) | 2.08 (0.43)* | 1.02 (0.18) |

Mean fatty acid levels (g/100 g fatty acids) ± SD, $n = 8$/group Means and standard deviations were calculated by One-way ANOVA followed by Bonferroni's post test (Graph Pad, Prism, Version 4.0) as compared to the Control group

*$p < 0.05$ compared to control

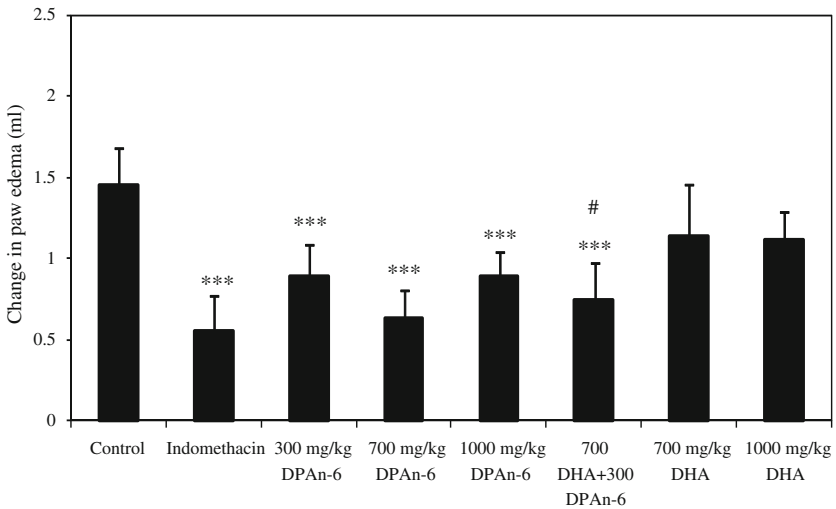


**Fig. 5** DHA/DPAn-6 combination reduces inflammatory edema in a rodent model of hind paw edema. A dose response study was performed to assess the effects of various dose levels of DPAn-6 on paw edema in this model. All groups of animals received an equivalent amount of fat which consisted of 300–1,000 mg/kg DHA or DPAn-6 per day, as indicated, with an appropriate amount of control oil to bring the total gavaged fat content to 1,000 mg/kg per day. Indomethacin was administered at 5 mg/kg i.p. 30 min prior to carrageenan challenge. Fatty acids were administered daily for 14 days prior to conducting the paw edema assay at 2 h post-carrageenan challenge. Means ($n = 8$) +/− SD. ***$p < 0.001$ compared to control. #$p < 0.05$ compared to 700 or 1,000 mg/kg DHA group

**Table 3** Plasma phospholipid fatty acid levels in DPAn-6 dose response study

| Fatty acid | Treatment groups (Total PUFA dose) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | DPAn-6 | | | DHA/DPAn-6 | DHA | | Indomethacin |
| | | 300 mg/kg | 700 mg/kg | 1,000 mg/kg | 1,000 mg/kg | 700 mg/kg | 1,000 mg/kg | |
| n-3 | | | | | | | | |
| 20:5n-3 EPA | 0.04 (0.06) | 0.14 (0.25) | 0.07 (0.07) | 0 (0) | 0.78 (0.17)* | 1.12 (0.18)* | 1.02 (0.14)* | 0 (0) |
| 22:6n-3 DHA | 1.74 (0.15) | 1.72 (0.07) | 1.6 (0.07) | 1.47 (0.15) | 5.85 (0.58)* | 6.16 (0.11)* | 6.48 (0.56)* | 1.65 (0.03) |
| n-6 | | | | | | | | |
| 20:4n-6 ARA | 21.25 (0.76) | 23.03 (1.27) | 24.37 (0.62)* | 24.4 (0.40)* | 18.37 (0.37) | 15.99 (1.01)* | 15.94 (2.04)* | 22.32 (0.53) |
| 22:5n-6 DPAn6 | 0.98 (0.12) | 3.28 (0.61)* | 4.95 (0.26)* | 6.33 (0.91)* | 0.8 (0.14) | 0.04 (0.04) | 0 (0) | 1.15 (0.07) |

Mean fatty acid levels (g/100 g fatty acids) +/− SD ($n = 3$ pooled samples/group). Means and standard deviations were calculated by One-way ANOVA followed by Bonferroni's post test (Graph Pad, Prism, Version 4.0)

*$p < 0.05$ as compared to the Control group

studies to date. Here, we demonstrate the anti-inflammatory activity of DHA-rich algal oils in vivo, particularly DHA-S oil, and provide the first evidence of the potent anti-inflammatory activity of the constituent omega-6 LC-PUFA, DPAn-6.

As an omega-6 fatty acid and as a product of ARA, it was unexpected to find anti-inflammatory activity associated with DPAn-6. However, another omega-6 PUFA, gamma linolenic acid (GLA; 18:3n-6) has been shown to have anti-inflammatory activity in vitro and in vivo [39]. GLA decreases levels of IL-1$\beta$ and TNF-$\alpha$ in LPS-stimulated PBMC and IL-1$\beta$ in LPS-stimulated human monocytes in vitro [40].

In vitro analyses showed that all three LC-PUFA (DHA, DPAn-6, and EPA) reduced the production of the pro-inflammatory cytokines IL-1$\beta$ and TNF-$\alpha$ in human PBMC, at concentrations as low as 10 μM (IL-1$\beta$). However, DPAn-6 appeared to be the most effective inhibitor in vitro, reducing levels of these pro-inflammatory cytokines, with observed relative potencies of DPAn-6 (most potent) > DHA > EPA. This finding correlates with another recent study that demonstrated the enhanced efficacy of DHA, as compared to EPA, in decreasing IL-1$\beta$, IL-6 and TNF-$\alpha$ production in LPS-stimulated THP-1 cells [8].

The consumption of dietary LC-PUFA can alter membrane phospholipids and the production of eicosanoids [11, 17–20]. Our studies show that in addition to decreasing levels of pro-inflammatory cytokines in vitro, DPAn-6 also decreased intracellular COX-2 levels by approximately 50% with a concomitant reduction in PGE2 production. PGE2 mediates acute inflammation, as it is a potent vasodilator and increases vascular permeability and edema [41, 42]. DHA had no effect on COX-2 expression, but PGE2 levels were reduced by DHA, suggesting that DHA may affect COX-2 enzyme activity or PGE synthase, which is also involved in the regulation of PGE2. Both COX-2 and PGE synthase are increased in cells in response to an inflammatory stimulus and the production of PGE2 may require both enzymes [43, 44]. In support of this hypothesis, Roman et al [45] showed a reduction in microsomal PGE synthase RNA in the presence of DHA in vitro, whereas EPA had no effect. Alternatively, DHA treatment may reduce PGE2 production as a result of reduced availability of the PGE2 precursor ARA.

In contrast, EPA and the DHA/EPA combination increased levels of COX-2 and PGE2 in PBMC in vitro. An upregulation of COX-2 has been previously described for EPA in human keratinocytes [46]. It is important to note, however, that the effects of DHA and EPA may be cell-specific and/or dependent on the type of inflammatory stimulus used, as others have described differential COX-2 modulation with these LC-PUFA [46–49].

The rat carrageenan paw edema model is a well-characterized acute model of innate immunity that is commonly used to evaluate anti-inflammatory activity of compounds [50–53]. The DHA-S oil was more effective than DHA-T oil when tested in this in vivo model and the major difference between the two algal oils is the presence of DPAn-6 (DHA-S oil). DPAn-6 treatment resulted in the largest reduction in edema volume, reaching 40–55% inhibition at lower doses. Maximal effects of DPAn-6 were also visible as early as 2 h post-carrageenan challenge, as compared to 4 h with DHA.

Interestingly, the DPAn-6 in vivo dose response resulted in a non-monotonic curve with the maximum effect observed with the 700 mg/kg dose. Lower and higher doses of DPAn-6 reduced inflammation to a lesser degree and their effects were limited to 2–4 h following the inflammatory stimulus. The lack of a standard dose response effect suggests that DPAn-6 might be converted to other fats in vivo, such as ARA. Tam et al [54] showed the retroconversion of DPAn-6 to ARA occurred when ARA levels were decreased by the presence of DHA. In further support of this, humans supplemented with the DHA-T oil

had a dose-dependent decrease in plasma ARA whereas higher doses of the DHA-S oil resulted in relatively higher levels of ARA [30].

Treatment with 1,000 mg/kg DHA ethyl ester resulted in three to fourfold increase in plasma DHA, as compared to control levels. Humans would require a dose of approximately 1.5–2 g DHA per day to achieve a similar increase in plasma DHA [30]. Analysis of plasma phospholipid levels clearly showed that a reduction of ARA levels did not correlate with anti-inflammatory activity, as plasma ARA levels remained high or are increased upon treatment with DPAn-6. We speculate that the ability of DPAn-6 to reduce both COX-2 and PGE2 levels, as seen in vitro, may contribute to the decreased inflammation seen in this COX-2-driven rat paw edema model. Our laboratory has also recently shown that DPAn-6 can be converted into oxylipins, resolvin-like molecules, with potent anti-inflammatory activity which could contribute to the observed reduction in inflammatory response in vivo following dosing with DPAn-6 [55]. Supplementation with DHA-S oil, containing DHA/DPAn-6 may reduce inflammation, raising levels of DHA, while maintaining ARA levels. Taken together, these results suggest that the DHA-S algal oil may be a novel anti-inflammatory supplement.

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