Effects of dietary supplementation with fish oil on in vivo production of inflammatory mediators in clinically normal dogs

Casey J. LeBlanc, DVM, PhD; David W. Horohov, PhD; John E. Bauer, DVM, PhD; Giselle Hosgood, BVSc, PhD; Glenna E. Mauldin, DVM, MS

Objective—To evaluate the effect of diets enriched with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on in vivo production of interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, prostaglandin E2 (PGE2), and platelet-activating factor (PAF) in dogs.

Animals—15 young healthy dogs.

Procedures—Dogs were randomly allocated to receive an isocaloric ration supplemented with sunflower oil (n = 5), fish oil (5), or fish oil plus vitamin E (5) for 12 weeks. At week 12, in vivo production of inflammatory mediators was evaluated in serum at multiple time points for 6 hours following stimulation with lipopolysaccharide (LPS).

Results—Serum activity or concentration (area under the curve) of IL-1, IL-6, and PGE2 significantly increased after LPS injection in all groups but to a lesser extent in dogs receiving the fish oil diet, compared with results for dogs receiving the sunflower oil diet. Serum activity of TNF-α and PAF concentration also increased significantly after LPS injection in all groups but did not differ significantly among groups.

Conclusions and Clinical Relevance—A fish oil–enriched diet consisting of 1.75 g of EPA/kg of diet and 2.2 g of DHA/kg of diet (dry-matter basis) with an n-6:n-3 fatty acid ratio of 3.4:1 was associated with significant reductions in serum PGE2 concentrations and IL-1 and IL-6 activities. Results supported the use of EPA- and DHA-enriched diets as part of anti-inflammatory treatments for dogs with chronic inflammatory diseases. Additional studies in affected dogs are warranted to further evaluate beneficial anti-inflammatory effects of EPA- and DHA-enriched diets. (Am J Vet Res 2008;69:486–493)

Cytokines are multifunctional peptides that are produced predominantly by activated lymphocytes and macrophages during immune and inflammatory reactions.1 Tumor necrosis factor-α, IL-1β, and IL-6 are among the most important cytokines produced by macrophages.2 Enhanced release of TNF-α, IL-1, and IL-6 is considered a hallmark of sepsis and hemorrhagic shock.3 These cytokines promote a multitude of changes, including fever; increased production of acute-phase proteins; hypotension; activation and infiltration of neutrophils; increased synthesis of cytokines by lymphocytes; and increased synthesis of lipid mediators, such as prostaglandins, leukotrienes, and PAF.4,5 These effects are crucial during the acute response against pathogens and injuries; however, overproduction of these cytokines can be severely detrimental to the host. Inappropriate or prolonged production has been associated with cartilage breakdown, organ dysfunction, anorexia and cachexia, and nonregenerative anemia.6,7

Cytokines are not the only important mediators of immunity and inflammation. Activation of cellular phospholipases leads to the synthesis of prostaglan-

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
</tbody>
</table>
dins, leukotrienes, and PAF. Prostaglandin E\(_2\) is an arachidonic acid metabolite produced predominantly by macrophages that is important in local acute inflammatory responses, such as swelling and erythema, and systemic inflammatory effects, especially fever.\(^\text{1,16}\) Platelet-activating factor is actually a family of structurally similar bioactive phospholipid-derived mediators.\(^\text{1,9}\) It is known that PAF has many proinflammatory effects on blood vessels, small vessels, and leukocytes, including increases in vascular permeability, bronchoconstriction, and adhesion and chemotaxis of neutrophils and eosinophils.\(^\text{10,11}\) Platelet-activating factor can enhance the synthesis of TNF-\(\alpha\) and IL-1 by stimulated macrophages.\(^\text{12,13}\)

Finding ways to inhibit the synthesis or block the biological effects of these inflammatory mediators is an active area of research. The ability to control these mediators offers the potential to minimize the negative impact of many chronic inflammatory diseases. However, commercially available anti-inflammatory medications can be expensive, and many have important toxic effects in humans and other animals, especially with long-term use. Dietary supplementation with \(n\)-3 fatty acids obtained from fish or fish oils has been suggested as a method to modulate the immune response while avoiding some of these toxic effects, and it appears to provide beneficial anti-inflammatory effects.

The principle \(n\)-3 fatty acids in fish or fish oils are EPA and DHA. Analysis of data suggests that dietary supplementation with these nutrients can influence the production of inflammatory mediators, which potentially could reduce the severity of some diseases. However, evaluations of the effects of dietary supplementation with \(n\)-3 fatty acids on these mediators in dogs are scarce and contradict results in other species. In 1 study,\(^\text{14}\) conducted by our laboratory group, we reported the effect of a diet enriched by the addition of EPA and DHA, with and without supplemental vitamin E, on selected hematologic and serum biochemical variables, serum concentrations of vitamin E, and lipid peroxidation in healthy young crossbred-hound dogs. In another study conducted by our laboratory group,\(^\text{15}\) lymphocyte proliferation of those same crossbred-hound dogs was significantly decreased in a group fed the same EPA- and DHA-enriched diet. The objective of the study reported here was to evaluate the effect of these same diets supplemented with EPA and DHA on in vivo production of IL-1, IL-6, TNF-\(\alpha\), PGE\(_2\), and PAF in similar healthy dogs.

### Materials and Methods

**Sample population**—Fifteen healthy crossbred-hound dogs were obtained from a colony for use in the study. The study population consisted of 8 sexually intact females, 6 sexually intact males, and 1 castrated male dog. Dogs ranged from 1 to 4 years of age. Body weight ranged from 9.5 to 12.3 kg, and each dog had a body condition score of 5 to 6 (scale of 1 to 9).\(^\text{16,17}\) on entry into the study. During the study, dogs were housed separately in covered outdoor runs and fed once daily. The experimental protocol was approved by the Louisiana State University Institutional Animal Care and Use Committee.

**Diets**—Prior to the experiment, each dog was fed a standard laboratory diet.\(^\text{4}\) During the course of the study, the experimental diet was specifically fed to meet energy requirements, as determined on the basis of the following equation\(^\text{18}\): metabolizable energy = \(132 \times (\) kg of body weight\(^\text{19}\)). The experimental population was randomly assigned to 1 of 3 dietary treatment groups for the duration of the 12-week study. Each dietary treatment group consisted of 5 dogs (3 female dogs and 2 male dogs or vice versa). The control group (sunflower oil group) received approximately 500 g of the standard laboratory diet supplemented with 12.4 g of sunflower oil and 3 to 5 g of a compressed portion of a low-calorie prescription diet.\(^\text{5}\) The diet of the control group was supplemented with sunflower oil to increase the n-6:n-3 ratio of fatty acids to be more consistent with most standard diets formulated for dogs. One treatment group (fish oil group) received approximately 500 g of the standard diet supplemented with 0.6 g of sunflower oil, 7 g of menhaden fish oil (1.65% oil on a dry-matter basis), and 3 to 5 g of a compressed portion of the low-calorie prescription diet.\(^\text{5}\) The other treatment group (fish oil plus vitamin E group) received the same diet as the other fish oil group, except 0.17 g of \(\alpha\)-tocopherol acetate was injected into the compressed portion of low-calorie prescription diet.\(^\text{5}\) This amount of supplemental vitamin E was above the minimum but below the maximum vitamin E concentration recommended for diets formulated for dogs.\(^\text{19}\) Dogs in the fish oil plus vitamin E group were provided the dietary supplement vitamin E to prevent a potential increase in lipid peroxidation secondary to consumption of a diet high in polyunsaturated fatty acids.\(^\text{14}\) The compressed portion of the low-calorie prescription diet was used as a vehicle for the supplemental vitamin E. All 3 dietary groups received the compressed portion of food to ensure similar caloric intake.

The standard laboratory diet (ie, kibble), sunflower oil, and menhaden fish oil were analyzed at a commercial laboratory.\(^\text{4}\) The composition of the kibble was 25.8% protein, 9.3% moisture, 8.9% crude fat, 2.3% crude fiber, and 7.2% ash (as determined on the basis of weight) and 46.5% nitrogen-free extract (on the basis of difference). Fatty acid composition and vitamin E concentration of the conventional and experimental diets were determined at the same commercial laboratory\(^\text{4}\) (Appendix).

**Experimental design**—After each group of dogs consumed the assigned experimental diet for 12 weeks, a sterile 16-gauge catheter was aseptically inserted in a jugular vein of each dog for the 6-hour experiment. Approximately 20 mL of blood was obtained through the catheter for use in analysis of circulating activities of IL-1, IL-6, and TNF-\(\alpha\) and concentrations of PAF and PGE\(_2\), at baseline (immediately before IV injection of LPS). Immediately prior to LPS injection was designated as time 0. Each dog was treated with an IV injection of LPS\(^\text{20}\) from *Escherichia coli* serotype O55:B5 in sterile saline (0.9% NaCl) solution at a dose of 0.1 \(\mu\)g/kg to stimulate the production of inflammatory mediators.\(^\text{20}\) Approximately 20 mL of blood was collected 0.5, 1, 1.5, 2, 3, 4, and 6 hours after the LPS injection for use in determining circulating blood concentrations of cytokines.
and lipid mediators of inflammation. During the 6-hour interval, dogs were monitored for signs of illness and rectal temperatures were measured hourly. After the 6-hour experimental period, 500 mL of lactated Ringer’s solution was administered IV to each dog for replacement of fluid loss, and the catheters were removed.

Serum bioassays for IL-1, IL-6, and TNF-α—Ten milliliters of blood (from the 20 mL collected at each time point) was placed into plain evacuated tubes, allowed to clot, and centrifuged to yield serum. Serum was harvested, separated into 0.5-mL aliquots, and frozen at –80°C until analysis for cytokine activity. These bioassays did not use specific antibodies, and the cytokines were not sequenced. The IL-1α, IL-6α, and TNF-αβ-like activities were measured on the basis of the proliferation or death of target cell lines.

Cell culture and preparation of cytokine standards—All 3 target cell lines used for the cytokine bioassay were cultured in 5% 1640 medium supplemented with 10% fetal calf serum, 2.0 mM L-glutamine, 2.2 g of NaHCO3/L, 25 mM HEPES, 2.7 × 10 –4 mg of 2-mercaptoethanol/L, 100 U of penicillin/mL, and 100 µg of streptomycin sulfate/mL. The B13.29 clone B9 cells used for the IL-6 bioassay were additionally supplemented with 75 U of recombinant murine IL-6/mL. Standard curves for each assay were generated by serial dilutions (1:3) of recombinant human IL-1α, IL-6, or TNF-αβ. Each recombinant cytokine promoted di- lution-dependent proliferation (or death) of the target cell line.

Bioassay for IL-1α—The cytoproliferative activity for IL-1α was performed with the human melanoma cell line A375.S2. Triplicate serum samples obtained at each time point were diluted 1:10 in 100 µL of culture medium and placed in 96-well flat-bottom plates. An additional 100 µL of culture medium containing 2 × 10 3 A375.S2 cells was placed in each well (final dilution of each serum sample, 1:20). Plates were sealed to prevent evaporation and incubated at 37°C in 5% carbon dioxide for 96 hours. After incubation, medium was removed and plates were washed once with calcium-and-magnesium–free PBS solution (pH, 7.2 to 7.4). The remaining cells were fixed by adding 50 µL of 100% methanol to each well and allowing the wells to incubate at 25°C for 30 minutes. Remaining methanol was then removed, and cells were stained by incubation with 50 µL of 0.5% crystal violet in 20% ethanol at 25°C for 30 minutes. Wells were thoroughly washed with tap water, and 100 µL of 50% methanol was added to each well to elute the incorporated violet stain. Cell survival was determined by measuring optical density of the plates at 595 nm in a multiple-well scanning spectrophotometer. Lower limit of detection of the assay for IL-1α was 7.4 U/mL.

Bioassay for IL-6α—Cytoproliferative activity of serum samples was assayed by use of the murine hybridoma IL-6β–dependent cell line B13.29 clone B9. Triplicate serum samples were diluted 1:100 with complete medium and placed in 96-well flat-bottom plates (100 µL/well). An additional 100 µL of medium containing 2 × 10 5 B9 cells was added to each well (final dilution of each serum sample, 1:200). Plates were sealed and incubated at 37°C in 5% carbon dioxide for 72 hours. For the final 4 hours of incubation, cells were pulsed with 1.0 µCi of tritiated thymidine/well. Cells were harvested onto glass fiber filters, and incorporation of tritiated thymidine was determined by use of liquid scintillation spectrophotometry. Lower limit of detection of the assay for IL-6α was 0.001 U/mL.

To determine whether the cytoproliferative activity was specific for IL-6, bioactivity was neutralized by incubating cells with a mouse anti-human CD126 (IL-6 receptor) IgG1 monoclonal antibody. Prior to the assay, B9 cells were incubated for 1 hour at 37°C at a concentration of 7.25 µg of antibody/2 × 10 5 cells.

Bioassay for TNF-αβ—Cytoproliferative activity of TNF-αβ against the mouse fibrosarcoma cell line WEHI 164 clone 13 was determined for triplicate serum samples. First, 96-well flat-bottom plates were seeded with 2 × 10 3 WEHI 164 clone 13 cells in 100 µL of complete medium/well and allowed to incubate overnight at 37°C in 5% carbon dioxide. Approximately 18 hours later, 55 µL of medium was removed from each well and discarded. This volume was replaced by 3 µL of actinomycin D (20 µg/mL) and 50 µL of serum (final dilution of each serum sample, 1:100). Plates were sealed and allowed to incubate at 37°C in 5% carbon dioxide. Approximately 20 hours later, 10 µL of tetrazolium dye 3-(4,5-dimethylthiazole-2-yl)-2,5 triphenyl tetrazolium bromide (5 mg/mL in calcium and magnesium–free PBS solution) was added to each well, and wells were incubated for an additional 4 hours at 37°C in 5% carbon dioxide. After incubation, 100 µL of 0.04 N HCl in isopropanol was vigorously pipetted into each well to dissolve any purple formazan crystals. After 30 minutes at 25°C, optical density of the plates was measured at 595 nm in a multiple-well scanning spectrophotometer. Lower limit of detection of the assay was 0.06 U/mL.

To determine whether cytokotoxicity was attributable to TNF-αβ, WEHI cells were incubated with rat anti-mouse CD120a (TNF-receptor 1) IgG2a monoclonal antibody. Prior to the assay, WEHI cells were incubated for 1 hour at 37°C at a concentration of 5 µg of antibody/2 × 10 5 cells.

Scintillation proximity assay for PAF—As a result of active acetylhydrolases in peripheral blood, PAF is quickly converted to an inactive metabolite, lyso-PAF. After collection, a 1.5-mL aliquot of blood was immediately extracted with 4.5 mL of ice-cold methanol to inactivate acetylhydrolase activity. Plasma was isolated by centrifugation, harvested, and frozen at –80°C until analysis. Semipurification of plasma was performed by use of a modified Bligh-Dyer lipid-phase extraction. Briefly, 3-mL samples were mixed with 1 mL of distilled water and 4 mL of chloroform. The aqueous phase was collected and evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in 200 µL of chloroform and applied to a C-18 minicolumn. The column was washed with 3 mL of chloroform, 2 mL of chloroform:methanol (3:2), and 3 mL of chloroform:methanol:28% aqueous ammonia (10:5:1) and then eluted with 2 mL of chloroform:methanol:28% aqueous ammonia (50:30:7). Eluate was evaporated under a stream of nitrogen gas, and the residue was dissolved in 200 µL of assay buffer.
The PAF concentration of the purified samples was determined for each time point with a scintillation proximity assay in accordance with the manufacturer’s instructions. All reagents were provided. Briefly, 100 µL of sample was combined with 100 µL of the tracer compound ([3H]-PAF), 100 µL of anti-PAF antibody, and 100 µL of scintillation proximity assay protein A (bound to fluomicrospheres). Each sample was prepared in duplicate and incubated on an orbital shaker for approximately 20 hours at 25°C. After incubation, the amount of [3H]-PAF bound to the fluomicrospheres was determined by an automated β-scintillation counter. Mean number of counts per minute was calculated for each replicate. The concentration of PAF within each sample was determined by use of a log-logit curve generated by inclusion of 7 standard dilutions. Lower limit of detection of the assay was approximately 20 pg/sample. Specificity of the assay for PAF (1-hexadecyl-2-acetyl glycerophosphocholine-PAF [C16:0]) was 100% with 40% cross-reactivity for PAF (1-octadecyl-2-acetyl glycerophosphocholine-PAF [C18:0]) but < 0.01% for lyso-PAF and other choline-containing phospholipids.

ELISA for PGE₂—Four milliliters of blood was added to an EDTA-containing evacuated tube with 10µM indomethacin to prevent conversion of PGE₂ to more stable metabolites. Plasma was isolated by centrifugation, harvested, and frozen at –80°C until analysis. Plasma PGE₂ concentrations were determined by use of a competitive ELISA for a stable PGE₂ derivative in accordance with the manufacturer’s instructions. Briefly, 500-µL aliquots of plasma were derivatized by adding 150 µL of 1M carbonate buffer and incubating overnight at 37°C. Buffered samples were further diluted to 1:10 and 1:20 and added in duplicate to wells of 96-well plates coated with goat anti-mouse IgG, which was provided by the manufacturer. Eight serial dilutions (1:2) of a provided standard were made and added to the appropriate wells. Next, 50 µL of tracer compound consisting of PGE₂ conjugated to acetylcholine was added to each well, followed by the addition of 50 µL of mouse anti-PGE₂. After incubation for 18 hours at 4°C, all wells were emptied and washed 5 times with buffer. After the addition of 200 µL of fresh acetylcholine and 2-nitrobenzoic acid/well, plates were allowed to develop on an orbital shaker for 90 minutes in the dark at 25°C. Intensity of the enzymatic reaction was determined spectrophotometrically at 415 nm. Sample PGE₂ concentrations were calculated by use of a log-logit curve generated by inclusion of 8 standard dilutions. Lower limit of detection of the assay was approximately 20 pg/mL. Specificity of the assay was 100%, with < 1% cross-reactivity with other eicosanoids.

Statistical analysis—All data were considered continuous and had a normal distribution, as determined by rejection of the null hypothesis of normality at P ≤ 0.05 by use of the Shapiro-Wilk test. For the 6-hour in vivo curves, the AUC (concentration vs time) was calculated by use of the trapezoidal method to enable in vivo curves, the AUC (concentration vs time) was calculated by use of the trapezoidal method to enable comparison among diet types. The AUC was evaluated by use of a mixed-effect linear model that accounted for the random variance of dog. When there was a significant (P ≤ 0.05) interaction between concentration and time, least square means were compared among and within diet groups to detect significant effects. Type I error was maintained at (α = 0.05) for all among- and within-group comparisons. Data were summarized as mean ± SEM. A Kruskal-Wallis test was used for the statistical analysis.

Results

During the 6-hour in vivo experiment, 3 of 5 dogs in group sunflower oil, 1 of 5 dogs in group fish oil, and 2 of 5 dogs in group fish oil plus vitamin E vomited between 1 and 2 hours after receiving the IV injection of LPS. Rectal temperatures for all dogs remained within the reference range during the experiment. All dogs appeared bright and alert and had a good appetite after receiving the fluids administered after completion of the experiment.

Immediately before LPS injection, there was no significant difference in serum cytokine activity or concentrations of PAF and PGE₂ among the 3 dietary groups. After IV injection of LPS, serum IL-1 activity (ie, AUC) of the 2 groups fed diets supplemented with fish oil was significantly lower (fish oil, P = 0.003; fish oil plus vitamin E, P < 0.001) than the serum IL-1 activity for the dogs fed the diet supplemented with sunflower oil (Figure 1). However, significant differences among groups at specific time points were only detected at 4 and 6 hours. The IL-1 activity of dogs in group sunflower oil was significantly higher than that for group fish oil (P = 0.002) at 4 hours and that for groups fish oil (P = 0.035) and fish oil plus vitamin E (P = 0.018) at 6 hours. Total IL-1 activity for groups fish oil and fish oil plus vitamin E did not differ significantly at any time point.

The IV injection of LPS stimulated a significant increase in serum IL-6 activity among all 3 dietary groups. Activity of IL-6 was significantly increased from baseline values at 1.5, 2, and 3 hours for all 3 dietary groups and at 4 hours for groups sunflower oil and fish oil plus vitamin E. Activity of IL-6 for group fish oil was significantly lower than activity for group sunflower oil at 1.5 (P = 0.006) and 4 (P = 0.013) hours. Evaluation of total serum IL-6 activity revealed that groups fish oil
Serum IL-6 activity for 3 canine dietary groups after IV administration of LPS. Total IL-6 activity (AUC) of the group receiving sunflower oil was significantly ($P < 0.05$) higher, compared with the total IL-6 activity for each of the 2 groups receiving fish oil. See Figure 1 for key. (Figure 2)

Serum TNF-α activity for 3 canine dietary groups after IV administration of LPS. Total TNF-α activity (AUC) did not differ significantly ($P > 0.05$) among groups. See Figure 1 for key. (Figure 3)

Serum PAF concentration for 3 canine dietary groups after IV administration of LPS. Total PAF concentration (AUC) did not differ significantly ($P > 0.05$) among groups. See Figure 1 for key. (Figure 4)

$P = 0.012$ and fish oil plus vitamin E ($P = 0.003$) were significantly lower than IL-6 activity for group sunflower oil. Total IL-6 activity for groups fish oil and fish oil plus vitamin E did not differ significantly at any time point (Figure 2).

Serum TNF-α activity of all 3 dietary groups was significantly increased 1 hour after IV injection of LPS. Activity remained significantly higher than the baseline value at 1.5 hours for group fish oil plus vitamin E. Significant differences among groups were not detected at any time point, including 1 and 1.5 hours after LPS injection. Total serum TNF-α activity did not differ significantly among the 3 dietary groups (Figure 3).

The IV injection of LPS induced significant changes in serum PAF concentration, compared with baseline values, in all 3 dietary groups. However, significant increases at 0.5, 1.5, and 2 hours did not differ among groups. Total PAF concentration did not differ significantly among the 3 dietary groups (Figure 4).

Significant increases of serum PGE$_2$ concentration, compared with baseline values, were detected at 0.5, 1, 1.5, and 2 hours after LPS injection for dogs in group sunflower oil. Dogs in group fish oil plus vitamin E had a significant increase in serum PGE$_2$ concentration at 1.5 hours after LPS injection. However, there were no significant increases in serum PGE$_2$ concentrations among dogs in group fish oil, compared with baseline concentrations. Increases in total serum PGE$_2$ concentrations of groups fish oil ($P = 0.002$) and fish oil plus vitamin E ($P = 0.018$) were significantly lower, compared with the PGE$_2$ concentration of group sunflower oil. Compared with serum PGE$_2$ concentrations for group sunflower oil, PGE$_2$ concentrations were significantly lower at 0.5, 1, 1.5, and 2 hours for groups fish oil ($P = 0.001, 0.016, 0.002$, and 0.028, respectively) and fish oil plus vitamin E ($P = 0.001, 0.007, 0.01$, and 0.024, respectively). Serum PGE$_2$ concentration did not differ significantly between groups fish oil and fish oil plus vitamin E (Figure 5).

**Discussion**

Studies in which investigators evaluated production of inflammatory mediators in clinically normal dogs fed diets supplemented with n-3 fatty acids have yielded mixed results. Maintaining young and older dogs on a diet consisting of 1.65% fish oil (n-6:n-3 ratio of 5:1) for 60 days had no effect on IL-1, IL-6, TNF-α, or PGE$_2$ production by peritoneal macrophages or peripheral blood mononuclear cells. In contrast, dietary supplementation with fish oil reduced PGE$_2$ production by peripheral blood mononuclear cells of dogs that received vitamin E. Significant differences among groups were not detected at any time point, including 1 and 1.5 hours after LPS injection. Total serum TNF-α activity did not differ significantly among the 3 dietary groups (Figure 3).

The IV injection of LPS induced significant changes in serum PAF concentration, compared with baseline values, in all 3 dietary groups. However, significant increases at 0.5, 1.5, and 2 hours did not differ among groups. Total PAF concentration did not differ significantly among the 3 dietary groups (Figure 4).

Significant increases of serum PGE$_2$ concentration, compared with baseline values, were detected at 0.5, 1, 1.5, and 2 hours after LPS injection for dogs in group sunflower oil. Dogs in group fish oil plus vitamin E had a significant increase in serum PGE$_2$ concentration at 1.5 hours after LPS injection. However, there were no significant increases in serum PGE$_2$ concentrations among dogs in group fish oil, compared with baseline concentrations. Increases in total serum PGE$_2$ concentrations of groups fish oil ($P = 0.002$) and fish oil plus vitamin E ($P = 0.018$) were significantly lower, compared with the PGE$_2$ concentration of group sunflower oil. Compared with serum PGE$_2$ concentrations for group sunflower oil, PGE$_2$ concentrations were significantly lower at 0.5, 1, 1.5, and 2 hours for groups fish oil ($P = 0.001, 0.016, 0.002$, and 0.028, respectively) and fish oil plus vitamin E ($P = 0.001, 0.007, 0.01$, and 0.024, respectively). Serum PGE$_2$ concentration did not differ significantly between groups fish oil and fish oil plus vitamin E (Figure 5).
ceived a diet consisting of 4.4% fish oil (n-6:n-3 ratio of 1.4:1) for 12 weeks. Production of leukotriene B\textsubscript{2}, another proinflammatory metabolite of arachidonic acid, was decreased in neutrophils and the skin of dogs fed diets with n-6:n-3 fatty acid ratios of 5.3:1 and 10:4:1, in which n-3 fatty acids comprised 3.4% and 1.8% of the total dietary fatty acids, respectively.\textsuperscript{27} A few clinical trials to assess the response to supplementation with n-3 fatty acids in dogs with naturally developing disease have also been reported. Pruritus associated with atopy was controlled in 8 of 18 (44.4%) dogs fed a commercial diet with an n-6:n-3 fatty acid ratio of 5:5:1.\textsuperscript{28} Other clinical trials in atopic dogs treated with n-3 fatty acids suggest beneficial effects in 5 of 45 (11.1%)\textsuperscript{29} and 17 of 93 (18.3%)\textsuperscript{30} dogs. Diminished signs of pain associated with hip dysplasia have been reported\textsuperscript{31} for dogs receiving supplemental n-3 fatty acids. Finally, dogs with heart failure were provided 27 mg of EPA/kg and 18 mg of DHA/kg each day for 8 weeks.\textsuperscript{32} Evaluation of components of circulating plasma and stimulated peripheral blood mononuclear cell cultures from those dogs revealed a significant decrease in mononuclear cell production of only IL-1; the TNF-\(\alpha\) activity and PGE\textsubscript{2} concentrations in those dogs did not differ significantly from values for control dogs.

Results of the study reported here are important because to our knowledge, they are the first for an in vivo measurement of LPS-stimulated production of cytokines and lipid mediators in dogs fed EPA- and DHA-enriched diets. Harvested macrophage cultures are an important tool for evaluating production of inflammatory mediators. However, most (if not all) inflammatory mediators are also produced by other cells, such as T lymphocytes, endothelial cells, mast cells, and fibroblasts.\textsuperscript{33} Although LPS-induced production of inflammatory mediators is predominantly from macrophages, contributions by other cell types cannot be discounted. Furthermore, measurement of in vivo mediator production also takes into account circulating half-lives and inhibitory mechanisms within a cell culture. Therefore, this in vivo method appears to be a useful tool for evaluating effects of EPA- and DHA-enriched diets on production of inflammatory mediators and addressing important issues not resolved by in vitro studies with conflicting results.

The study reported here was a continuation of a research project conducted to evaluate young healthy dogs fed a diet supplemented with menhaden fish oil, which consisted of 1.75 g of EPA/kg of diet (dry-matter basis), 2.2 g of DHA/kg of diet DHA (dry-matter basis), and a dietary n-6:n-3 fatty acid ratio of 3:4:1. In another study\textsuperscript{14} conducted by our laboratory group, it was reported that consumption of this diet did not significantly increase plasma concentrations of lipid peroxide or diminish serum vitamin E concentrations. In a subsequent report,\textsuperscript{15} there was diminished lymphocyte proliferation and macrophage production of PGE\textsubscript{2} in these dogs consuming the same EPA- and DHA-enriched diet. In the study reported here, the effect of this diet (with and without vitamin E supplementation) was evaluated on in vivo production of IL-1, IL-6, TNF-\(\alpha\), PGE\textsubscript{2}, and PAF. Analysis of our results revealed that feeding a diet with this quantity and an n-6:n-3 ratio of fatty acids had significant effects on the synthesis of several inflammatory mediators in young adult dogs.

Total serum activity (ie, AUC) for each cytokine in each dietary group peaked after LPS administration at time points similar to those in other reports.\textsuperscript{25,34} Total serum IL-1 activity of the 2 groups fed diets supplemented with fish oil was significantly lower for the control group fed a diet supplemented with sunflower oil. However, significant differences among the 3 dietary groups at specific time points were not apparent until 4 and 6 hours. Total serum IL-1 activity for group sunflower oil did not significantly increase from the baseline value until 4 and 6 hours after LPS injection. This delayed increase in activity was unexpected on the basis of another study.\textsuperscript{35} It is possible that cytotoxic effector cells could be involved in IL-1 activity. Therefore, the actual total serum IL-1 activity may not have differed significantly among groups.

In contrast to TNF-\(\alpha\), IL-6 is synthesized by many cell lines, including macrophages, endothelial cells, fibroblasts, and activated T lymphocytes, in response to endotoxin and other cytokines, such as TNF and IL-1.\textsuperscript{36} In another study,\textsuperscript{37} total serum IL-6 activity after IV injection of LPS was significantly lower in dietary groups fed diets supplemented with fish oil, compared with results for dogs fed a diet supplemented with sunflower oil, even though there was no difference among the groups for TNF-\(\alpha\) activity. This implied that dietary supplementation with n-3 fatty acids may have attenuated direct induction of IL-6 synthesis by LPS. In the study reported here, total serum TNF-\(\alpha\) activity did not differ significantly among the 3 dietary groups. This is consistent with results for other studies\textsuperscript{38,39} in dogs that failed to detect a significant effect of dietary n-3 fatty acids on mononuclear cell production of TNF-\(\alpha\). Addition of an anti-mouse TNF-\(\alpha\) receptor antibody failed to inhibit target cell death induced by human recombinant TNF-\(\alpha\). The affinity of the antibody may not have been sufficient to inhibit the cytokine from binding to the receptor and preventing cell killing. Second, binding of the antibody to the receptor may not have inhibited binding of TNF-\(\alpha\) to the receptor. Nevertheless, because the human recombinant TNF-\(\alpha\) used in the study consistently caused dilution-dependent cell death of the WEHI cell line, the assay should have been appropriate for detecting TNF-\(\alpha\) activity in canine samples.

Dietary supplementation with n-3 fatty acids can suppress PAF synthesis in other species.\textsuperscript{40–42} Serum PAF concentrations did not differ significantly among the 3 dietary groups in the study reported here.

Several studies\textsuperscript{43–45} in humans and other animals have revealed significant reductions in PGE\textsubscript{2} synthesis by mononuclear cells following supplementation with n-3 fatty acids. The study reported here also yielded significantly lower PGE\textsubscript{2} concentrations in serum following LPS stimulation of dogs consuming diets enriched with EPA and DHA, compared with PGE\textsubscript{2} concentrations of dogs consuming the control diet (group sunflower oil). This effect of dietary supplementation with n-3 fatty acids on systemic concentrations of PGE\textsubscript{2} was expected on the
basis of the established relationship between fatty acids and prostaglandin production.13,14

Direct influence of vitamin E or lipid peroxidation on cytokine production is rarely addressed.15 Low amounts of vitamin E may allow increases in lipid peroxidation, which could inhibit lymphocyte or macrophage function, including cytokine production. Significant differences in cytokine production were not detected between the 2 groups consuming fish oil but with differences in their concentrations of dietary vitamin E. However, this was not surprising considering that neither group had increases in plasma concentrations of lipid peroxides or decreases in concentrations of vitamin E.16

A diet enriched by the addition of menhaden fish oil, which consisted of 1.75 g of EPA/kg of diet and 2.2 g of DHA/kg of diet with an n-6:n-3 fatty acid ratio of 3.4:1, was associated with significantly lower serum PGE2, concentrations and IL-1 and IL-6 activities. Analysis of results of the study reported here, along with results of another study,15 in which effects on lymphocyte proliferation were evaluated, appears to support the use of EPA- and DHA-enriched diets as part of an anti-inflammatory arsenal against chronic inflammatory diseases in dogs. Additional studies in affected animals are warranted to further evaluate the beneficial anti-inflammatory effects of such EPA- and DHA-enriched diets.

References


**Appendix**

Concentration of selected fatty acids and vitamin E in the experimental diets (dry-matter basis)* fed to 3 groups of dogs (5 dogs/dietary group) for 12 weeks.

<table>
<thead>
<tr>
<th>Component</th>
<th>Basal diet</th>
<th>Sunflower oil</th>
<th>Fish oil</th>
<th>Fish oil plus vitamin E</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:1 (n-7)</td>
<td>2.6</td>
<td>2.9</td>
<td>4.4</td>
<td>4.4</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>33.8</td>
<td>43.9</td>
<td>37.9</td>
<td>37.9</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>20.7</td>
<td>39.8</td>
<td>23.0</td>
<td>23.0</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>22:4 (n-6)</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>18:3 (n-3)</td>
<td>1.1</td>
<td>1.2</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>18:4 (n-3)</td>
<td>0.2</td>
<td>0.3</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>20:4 (n-3)</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>20:5 (n-3)</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>22:5 (n-3)</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>22:6 (n-3)</td>
<td>0.1</td>
<td>0.1</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Sum of saturated fatty acids</td>
<td>30.4</td>
<td>36.7</td>
<td>36.7</td>
<td>36.7</td>
</tr>
<tr>
<td>Sum of monounsaturated fatty acids</td>
<td>37.2</td>
<td>47.7</td>
<td>42.9</td>
<td>42.9</td>
</tr>
<tr>
<td>Sum of polyunsaturated fatty acids</td>
<td>22.5</td>
<td>41.8</td>
<td>31.4</td>
<td>31.4</td>
</tr>
<tr>
<td>Sum of unsaturated fatty acids</td>
<td>59.7</td>
<td>89.5</td>
<td>74.4</td>
<td>74.4</td>
</tr>
<tr>
<td>Sum of all fatty acids</td>
<td>90.1</td>
<td>126.1</td>
<td>111.1</td>
<td>111.1</td>
</tr>
<tr>
<td>Sum of n-6 fatty acids</td>
<td>21.1</td>
<td>40.1</td>
<td>23.7</td>
<td>23.7</td>
</tr>
<tr>
<td>Sum of n-3 fatty acids</td>
<td>1.4</td>
<td>1.7</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>n-6:n-3 ratio</td>
<td>14.7</td>
<td>24.0</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>40.3</td>
<td>79.7</td>
<td>58.4</td>
<td>493.3</td>
</tr>
</tbody>
</table>

*Values reported are grams of component per kilogram of diet, except for vitamin E, which is reported as the units of vitamin E per kilogram of diet.

*Analysis performed at a commercial laboratory.*
