N-3 polyunsaturated fatty acids do not affect cytokine response to strenuous exercise

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Toft, Anders Dyhr, Mette Thorn, Kenneth Ostrowski, Sven Asp, Kirsten Møller, Susanne Iversen, Claus Hermann, Sisse Rye Søndergaard, and Bente Klarlund Pedersen. N-3 polyunsaturated fatty acids do not affect cytokine response to strenuous exercise. J Appl Physiol 89: 2401–2406, 2000.—The aim of the present study was to investigate whether fish oil supplementation was able to modulate the acute-phase response to strenuous exercise. Twenty male runners were randomized to receive supplementation (n = 10) with 6.0 g fish oil daily, containing 3.6 g n-3 polyunsaturated fatty acids (PUFA), for 6 wk or to receive no supplementation (n = 10) before participating in The Copenhagen Marathon 1998. Blood samples were collected before the race, immediately after, and 1.5 and 3 h postexercise. The fatty acid composition in blood mononuclear cells (BMNC) differed between the fish oil-supplemented and the control group, showing incorporation of n-3 PUFA and less arachidonic acid in BMNC in the supplemented group. The plasma levels of tumor necrosis factor-α, interleukin-6, and transforming growth factor-β1 peaked immediately after the run, the increase being 3-, 92-, and 1.1-fold, respectively, compared with resting samples. The level of interleukin-1 receptor antagonist peaked 1.5 h after exercise, with the increase being 87-fold. However, the cytokine levels did not differ among the two groups. Furthermore, supplementation with fish oil did not influence exercise-induced increases in leucocytes and creatine kinase. In conclusion, 6 wk of fish oil supplementation had no influence on the acute-phase response to strenuous exercise.

interleukin; sport; muscle

STRENuous exercise induces an acute-phase response that has similarities to that observed in sepsis and trauma (31), including increased plasma concentrations of interleukin (IL)-6 (26), IL-1 receptor antagonist (IL-1ra) (30), and tumour necrosis factor-α (TNF-α) (13, 29). Sepsis and trauma are associated with increased plasma levels of transforming growth factor (TGF)-β1 (18, 21–23, 38). TGF-β1 is an anti-inflammatory cytokine that represses the production of inflammatory cytokines by activated macrophages (4) and induces the release of soluble TNF receptors and IL-1ra (39). The effect of exercise on this cytokine has not been investigated.

The n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid and docosahexaenoic acid, both found in fish oils, suppress the production of arachidonic acid, derived 2-series prostaglandins, and the 4-series leukotrienes (16) that modulate the production of proinflammatory and immunoregulatory cytokines (7). In addition, eicosapentaenoic acid is a substrate for the synthesis of an alternative family of eicosanoids, the 3-series prostaglandins and the 5-series leukotrienes (5).

Dietary fats that are rich in n-3 PUFA have the potential to alter cytokine production. Animal studies indicate that n-3 PUFA-rich oils reduce the response to endotoxin and to proinflammatory cytokines (7). Thus these studies provide evidence that feeding plant or fish oils that are rich in n-3 PUFA alters the ex vivo production of TNF-α, IL-1, IL-2, and IL-6 (3, 19, 20, 37); however, contradictory observations do exist. Human studies provide more consistent data. Several studies have shown that supplementation of the diet of healthy volunteers with fish oils results in reduced production of IL-1, IL-6, TNF-α, and IL-2 by peripheral blood mononuclear cells (BMNC) in vitro (12). There are no human studies examining in vivo cytokine production after supplementation with n-3 PUFA.

Other studies examined the influence of n-3 PUFA in exercising humans, finding no difference in endurance performance (27, 32), no major changes in acute phase reaction proteins (15), and unchanged red blood cell deformability (27). Aguilaniu et al. (1, 2) investigated the exercise-related hypoxemia that was reduced in n-6 PUFA supplemented athletes (1, 2).

Changes in in vitro cell function induced by n-3 PUFA supplementation to humans have been intensively studied, finding reduced 4-series leukotrienes production by neutrophils, reduced neutrophil (14) and monocyte (35) chemotaxis to several agents, and inhib-
itiated neutrophil superoxide production (9, 41), although the last point is controversial (17).

In this experiment, exercise is used as a model of inflammation to study the effects of dietary supplementation with n-3 PUFA on in vivo cytokine production during strenuous exercise.

MATERIALS AND METHODS

Subjects. Twenty healthy, endurance-trained, volunteer athletes (men) completed the Copenhagen Marathon Race on May 18, 1998. None of the subjects was taking any medication. The experimental protocol was approved by the local ethics committee of the Copenhagen and Frederiksberg Communities. All subjects were informed about the purpose and risks of the study before their written, informed consent was obtained. Ambient temperature during running was 19°C.

For each subject, maximal oxygen consumption ($\dot{V}O_2$ max) was determined before the experiment during an incremental exercise test on treadmill.

Study design. Twenty men who were endurance-trained runners were randomized to receive supplementation ($n = 10$) of fish oil in capsules (Pikasol, Lube) 6.0 g daily, containing 3.6 g of n-3 PUFA (53% eicosapentaenoic acid and 31% docosahexaenoic acid) and 21.6 mg tocopherol for 6 wk or to receive no supplementation (control group; $n = 10$) before participating in The Copenhagen Marathon 1998.

The subjects were instructed to consume carbohydrate-rich foods (>8 g carbohydrate per kg of body mass per day) and fluids for 6 wk before the experiment, as well as 2 days after.

The subjects prepared for the preexercise sample in a way similar to their preparations for the marathon race. This preparation involved refraining from exercise for 2 days before the preexercise sample was obtained.

Sampling of blood and preparation of plasma, serum, and cells. Blood samples were drawn from the antecubital vein one week before the race, immediately after (median: 10 min, range: 2–23 min), and 1.5 and 3.0 h postexercise. The preexercise sample was taken 1 wk before the race to avoid influencing the performance of the runners. The subjects were kept at rest for 3 h postexercise, during which postexercise samples were taken.

For cytokine measurements, two 10-ml blood samples were drawn into glass tubes containing 35 $\mu$mol dipotassium-EDTA and 1,500 kallikrein inactivator units Trasylol (Bayer, Leverkusen, Germany). The tubes were kept on ice until they were centrifuged at 2,500 $g$ for 15 min at 4°C. Serum was isolated from blood samples drawn into endotoxin-free tubes without EDTA. Plasma and serum were stored at −80 and −20°C respectively.

For BMNC membrane phospholipid analysis after the supplementation period, a 20-ml blood sample was drawn into a glass tube containing heparin (nonconserved; 100 $\mu$m, 5,000 IE/ml; SAD). BMNC were isolated by density-gradient centrifugation (Lymphoprep, Nycomed Pharma, Oslo, Norway) on LeucoSep tubes (Greiner, Frickenhausen, Germany) and washed three times with stock solution I and Hanks’B (Bie and Berntsen AS, Redovre, Denmark) solution. BMNC were frozen in freezing medium and kept in liquid nitrogen until thawed for analysis.

Measurement of cytokines. TNF-α, IL-6, and IL-1α were measured in plasma. TGF-β1 was measured in serum. Cytokines were analyzed by commercially available enzyme-linked immunosorbent assay (ELISA; R & D Systems, Minneapolis, MN). All measurements were performed in duplicate, and high-sensitivity kits were used when available (which was the case for IL-6 and TNF-α but not for IL-1ra and TGF-β1). According to information provided by R & D Systems, the ELISA used for measuring IL-6 and TNF-α are insensitive to the addition of the recombinant forms of the soluble receptors (sIL-6r, sTNF-r1, and sTNF-r2, respectively), and these measurements, therefore, correspond to both soluble and receptor-bound cytokine. For the cytokine ELISA, the detection limits were IL-6 < 0.004, TNF-α < 0.18, IL-1α < 22, and TGF-β1 < 7, all in pg/ml. The intra-assay coefficients of variation were IL-6 = 6.0%, TNF-α = 4.9%, IL-1α = 6.2% and TGF-β1 = 5.3%.

Correction for plasma volume shifts. Changes in plasma volume caused by dehydration were calculated from measurements of hemoglobin and hematocrit, according to the method described by Dill and Costill (10). Cytokine, creatine kinase (CK), and leucocyte measurements were corrected accordingly, with a median correction factor of 0.96 (range: 0.72 to 1.1) immediately after the race.

Clinical chemistry analysis. All clinical chemistry analyses were performed by the Central Laboratory at the University Hospital of Copenhagen. Measurements of hemoglobin, hematocrit, lymphocytes, and neutrophils were done by a cell counter (SE-9000, Toa-Sysmex, Hamburg, Germany). CK was measured using automated enzyme reactions (Hitachi Systems 717, Boehringer Mannheim Diagnostica).

BMNC lipid analysis. The thawed cell pellets were washed three times with isotonic saline, suspended in 1 ml methanol with 0.002% 2,6-di-tert-butyl-p-cresol, and sonicated for 45 s. Lipids were then extracted quantitatively with 3 ml of 0.002% BHT in methanol-chloroform (2:1 ratio), followed by 2 ml of methanol-chloroform (1:1 ratio). The pooled supernatants were evaporated to dryness, dissolved in 540 $\mu$l of ether-methylacetate-sodium methoxide in methanol (25:1:1 M ratio), and vortexed for 10 s. After a 5-min reaction time, the mixtures were neutralized with acetic acid, evaporated to dryness, and the methyl esters were extracted by addition of 1 ml heptane and 200 $\mu$H$_2$O.

Separation of fatty acid methyl esters was performed on a HP6890 chromatograph (Hewlett-Packard, Wilmington, DE) fitted with a 50-m fused silica capillary column (CPSil88, 0.25 mm ID, film thickness 0.20 $\mu$m; Chrompack, Middelburg, The Netherlands), with a temperature rise from 150 to 220°C at 4°C/min. The injection temperature was 250°C, and the detector temperature was 270°C. Helium was used as the carrier gas, at 33 psi, with a 1- to 10 split ratio. PUFA were identified by comparison with authentic standards (Nu Chek Prep, Elysian, MN), and the results are expressed as percentage by weight.

Statistical analysis. The measurements of cytokines, leucocytes, and CK were tested for effects of time and group in a repeated-measures ANOVA. The model used was

\[
[\text{Measurement}] = \text{constant} + \text{time} + \text{group} + \text{error}
\]

Before proceeding with the statistical analysis, the residuals in the repeated-measures ANOVA were examined for a normal distribution, through investigation of a histogram and a normal plot. If residuals were considered abnormally distributed, positively skewed data were log-transformed, and residuals were investigated again. This was the case for IL-6, IL-1ra, lymphocytes, and CK.

If the effect of time tested significant ($P < 0.05$), a paired $t$-test was performed to compare postexercise measurements with preexercise values. $P$ values were adjusted using the Bonferroni method. Correlation analysis was performed using Pearson’s correlation coefficients. BMNC lipid data were analyzed by ANOVA and Duncan’s multiple-range test.
RESULTS

The two groups did not differ regarding age, height, weight, $V_O^2$max, and racing time (Table 1).

**Effect of exercise.** Strenuous exercise induced increases in the plasma concentration of TNF-$\alpha$ (3-fold), IL-6 (92-fold), IL-1ra (87-fold), and TGF-$\beta_1$ (1.1-fold) ($P < 10^{-10}$ for TNF-$\alpha$, IL-6, and IL-1ra; $P < 10^{-4}$ for TGF-$\beta_1$; Fig. 1). Maximal levels of TNF-$\alpha$, IL-6, and TGF-$\beta_1$ were found immediately after the race, whereas IL-1ra peaked 1.5 h afterward. Cytokine levels declined during the resting period and remained significantly different from the prerace values until at least 3 h after running (Fig. 1). Peak IL-6 was not correlated to peak CK ($r = 0.3; P = 0.2; n = 19$).

The lymphocyte levels were unchanged immediately after the race compared with prerace values. However, 1.5 h postexercise, the lymphocyte concentration had declined to $\sim 50\%$ of the prerace value ($P < 10^{-5}$; Fig. 2A). The neutrophil concentration was increased fivefold immediately after the exercise compared with prerace values and remained unchanged at 1.5 h postexercise ($P < 10^{-10}$, Fig. 2B). CK peaked the day after the race with a 17-fold increase and declined the following day ($P < 10^{-10}$; Fig. 2C).

**Effect of supplementation.** Comparing the n-3 PUFA-supplemented group with the control group, there were no differences in cytokine levels, neutrophil and lymphocyte concentrations, or CK levels (see Figs. 1 and 2).

Statistically significant differences between the fish oil-supplemented and control groups were observed for the fatty acids (20:4 n-6 arachidonic acid, 20:5 n-3 eicosapentaenoic acid, and 22:5 n-3) and, consequently, for the sums $\Sigma$n-6 PUFA, $\Sigma$n-3 PUFA, and the $\Sigma$n-6 PUFA-to-$\Sigma$n-3 PUFA ratio ($P < 0.01$), with all showing incorporation of n-3 PUFA and less arachidonic acid in BMNC than the supplemented group. No differences were observed among the other fatty acids (Table 2).

**DISCUSSION**

The present study showed that strenuous exercise induces significant increases in TNF-$\alpha$, IL-6, and IL-1ra, and these findings support previous observations (8, 11, 26, 28–30, 33, 36, 40). Plasma levels of TGF-$\beta_1$ have been shown to increase in response to sepsis (18, 23) and trauma (21, 22, 24). Exercise induced a small but significant increase in the plasma concentration of this cytokine. We are not aware of any other studies investigating the influence of exercise on TGF-$\beta_1$. Sepsis, trauma, and exercise seem to induce similar effects on TGF-$\beta_1$, even though the exercise-induced increase was only 10% compared with the twofold increase observed in response to sepsis (23). In contrast, when comparing the TNF-$\alpha$ and IL-6 responses to those of severe pneumococcal infections (6) and of a marathon (29, 30), almost identical cytokine levels were found.

A previous study, which used an eccentric exercise model (5), showed that IL-6 was associated with mus-
cle damage. However, in the present running model, we were unable to find any association with muscle damage, as visualized by increased levels of CK and peak IL-6.

The present study indicates that exercise induces production or release of cytokines by mechanisms other than that seen in sepsis. Thus, in other experimental models in which n-3 PUFA supplementation had an effect, cytokine production was stimulated by endotoxins (lipopolysaccharide). It has not been previously shown that exercise induces an increase in cytokine production through endotoxins. The latter mechanism is likely to play a role only in extreme exercise situations and to only be partly responsible for the exercise-associated increase in cytokines (25).

A main purpose of this study was to evaluate whether n-3 PUFA supplementation affected the acute-phase response to exercise. However, the present study clearly showed that n-3 PUFA supplementation for 6 wk did not induce any effects on plasma levels of TNF-α, IL-6, IL-1ra, or TGF-β1. Furthermore, we found no effects on neutrophil and lymphocyte concentrations and no influence on muscle damage, as visualized by the plasma CK level.

An alternative explanation for the contrasting findings regarding n-3 PUFA influences on cytokine levels between a previous animal study (7) and the present human study is that much higher doses of fish oil and longer supplementation periods were used in the animal experiment. In this study, doses of n-3 PUFA were sixfold higher than the daily dietary dose recommended by The European Union’s Scientific Committee for Food (34). Furthermore, PUFA analyses showed that we were able to identify two groups that differed with respect to the amount of n-3 PUFA in BMNC plasma membranes. Thus significant differences were observed for the fatty acids (20:4 n-6 arachidonic acid and 20:5 n-3 eicosapentaenoic acid) in the fish oil-supplemented group compared with controls. These fatty acids are precursors to different series of eicosanoids. This finding strongly indicates that the supplementation period and the amount of fish oil were sufficient for our purposes, and that fish oil supplementation is not likely to have any effect on exercise-induced cytokine production, even if given in larger doses or for longer time period.

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**Table 2. Percentage composition of BMNC fatty acids obtained from control and supplemented subjects**

<table>
<thead>
<tr>
<th>FA</th>
<th>Controls (n = 7)</th>
<th>Fish Oil (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>16.9 ± 0.6</td>
<td>17.1 ± 0.7</td>
</tr>
<tr>
<td>18:0</td>
<td>18.6 ± 0.9</td>
<td>19.0 ± 0.5</td>
</tr>
<tr>
<td>18:3</td>
<td>17.4 ± 1.2</td>
<td>17.7 ± 0.9</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>8.8 ± 1.5</td>
<td>8.2 ± 0.7</td>
</tr>
<tr>
<td>20:3 n-6</td>
<td>2.1 ± 0.3</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>20:4 n-6, AA</td>
<td>20.6 ± 1.4</td>
<td>18.3 ± 1.3*</td>
</tr>
<tr>
<td>20:4 n-3</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>20:5 n-3, EPA</td>
<td>0.9 ± 0.3</td>
<td>2.5 ± 0.7*</td>
</tr>
<tr>
<td>22:4 n-6</td>
<td>2.1 ± 0.5</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>22:5 n-6</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>22:5 n-3</td>
<td>3.3 ± 0.5</td>
<td>4.7 ± 0.6*</td>
</tr>
<tr>
<td>22:6 n-3, DHA</td>
<td>4.0 ± 1.0</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>Σ Minor FA</td>
<td>4.4 ± 0.3</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>Σ Unidentified peaks</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Σ SFA</td>
<td>36.4 ± 0.7</td>
<td>36.9 ± 0.7</td>
</tr>
<tr>
<td>Σ MUFA</td>
<td>20.0 ± 1.2</td>
<td>20.1 ± 1.0</td>
</tr>
<tr>
<td>Σ PUFA</td>
<td>43.2 ± 0.9</td>
<td>42.7 ± 1.0</td>
</tr>
<tr>
<td>Σ PUFA n-6</td>
<td>34.6 ± 1.6</td>
<td>30.2 ± 1.7*</td>
</tr>
<tr>
<td>Σ PUFA n-3</td>
<td>8.4 ± 1.6</td>
<td>12.4 ± 1.5*</td>
</tr>
<tr>
<td>Σ PUFA n-6Σ PUFA n-3</td>
<td>4.3 ± 1.2</td>
<td>2.5 ± 0.5*</td>
</tr>
</tbody>
</table>

Values are means ± SD. FA, fatty acids; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; Σ, sums of FA; Σ PUFA n - 6Σ PUFA n - 3, ratio of Σ PUFA n - 6 to Σ PUFA n - 3. *P < 0.01 compared with controls.
In conclusion, the present study found that strenuous exercise enhances the plasma levels of TNF-α, IL-6, IL-1β, and TGF-β1 and that 6 wk of fish oil supplementation did not affect exercise-induced cytokine levels, despite n-3 PUFA being incorporated into BMNC plasma membranes.

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