The Effect of Oral Administration of Eicosapentaenoic and Docosahexaenoic Acids on Acute Inflammation and Fatty Acid Composition in Rats

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Summary To investigate effects of eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) on acute inflammation, we fed rats either of the following four diets: an EPA-rich diet for 5 or 15 days, a DHA-rich diet for 5 or 15 days, a control diet for 5 or 15 days, and standard chow for 15 days. At the end of diets, the carrageenan-induced swelling of footpads was measured. Peritoneal cells were analyzed for their fatty acids in the phospholipid fraction. The swelling was similarly reduced in the EPA and DHA groups ($p < 0.05$, if fed for 15 days) compared with rats fed the control diet for 15 days. The mean proportion of arachidonic acid (AA) to the sum of highly unsaturated fatty acids was correlated ($r = 0.87$) to the mean degree of swelling among all dietary groups ($n = 7$). Effects of EPA and DHA might be explained by the reduced availability of AA for eicosanoid formation represented by the proportion of AA.

Key Words acute inflammation, carrageenan, eicosapentaenoic acid, docosahexaenoic acid, *n*-3 fatty acids, fatty acid composition, peritoneal cells, arachidonic acid

n-3 highly unsaturated fatty acids with 20 or 22 carbons (HUFA), which are abundant in marine oils, have been known to have beneficial effects on atherosclerotic disease (1). Those fatty acids have also been suggested to be beneficial in certain chronic inflammatory disease such as rheumatoid arthritis (2) and psoriasis (3). It is interesting to note that *n*-3 HUFA are beneficial for both of these two entirely different categories of disease. However, that is quite understandable by taking into consideration that the two categories have one major common denominator, eicosanoids, as a major disease modifier. Indeed, depression of eicosanoid production by *n*-3 HUFA is well-known (1, 4).

Since eicosanoids are also intimately associated with acute inflammation, oral

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administration of *n*-3 fatty acids may be beneficial for acute inflammation. In fact, degree of the acute inflammation experimentally induced by carrageenan is reported to be reduced by oral administration of *n*-3 HUFA in advance (5).

In the present study, we compared anti-inflammatory effects between purified eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) and investigated if the changes in fatty acid composition after *n*-3 HUFA administration could help understand the anti-inflammatory mechanisms of *n*-3 HUFA.

MATERIALS AND METHODS

HUFA and diets. EPA and DHA ethyl esters were obtained as previously described (6, 7) with slight modifications. Their purity was over 97% each. Soybean oil was purchased from a local market and transesterified (6). These three ethyl esters were fortified with 0.2% DL- α -tocopherol (Sigma, St Louis, MO) and 0.02% 2-*tert*-butylhydroquinone (Sigma) to prevent peroxidation of oils before mixing dietary components. A lipid-free powder diet was a product of Funabashi Farm (Funabashi). Nine hundred grams of the powder diet was mixed with 80 g of lard (purchased from a local market) and 20 g of EPA ethyl ester for the EPA diet, or 20 g of DHA ethyl ester for the DHA diet. For the high-fat control diet, 20 g of transesterified soybean oil was added instead of *n*-3 fatty acids. The fatty acid composition of these three mixed diets is shown in Table 1. The high-fat diets (the EPA, DHA, and high-fat control diets) were pelleted, slightly γ -radiated and sealed in small plastic bags without oxygen by Funabashi Farm. The bags were stored in a dark cold room. Diet of opened bags was used with 2 days, and all high-fat diets were used up within 3 weeks of manufacture. In this way, peroxide values of diets were kept low (less than 50 meq/kg of diet after 2 days in the open air at 25°C). A standard chow for rats (CE-2) was purchased from Clea Japan Inc. (Tokyo) and used as the reference diet. The fatty acid composition of CE-2 is also shown in Table 1.

Study design. Male Wistar rats were purchased from Sankyo Lab Service (Tokyo), and entered the study when they were 5 weeks of age (day 0). They had been on the standard CE-2 chow before the entry to the study. Seven dietary groups ($n=5$ for each group) were studied. They were fed as shown in Fig. 1. The EPA, DHA, or high-fat control diets were given to 3 groups of rats for 15 days (the EPA-15, DHA-15, and HF-15 groups, respectively). We used the HF-15 group as the control. Another 3 groups of rats continued to be fed the standard chow until day 10, then the chow was switched to either the EPA, DHA, or high-fat control diet for 5 days until day 15 (the EPA-5, DHA-5, and HF-5 group, respectively). Rats in the last group were fed the standard chow throughout the experimental period and were used for reference (the REF group). All rats had free access to their respective diets and tap water. On day 15 the right hind foodpad of rats of the seven groups was injected with 0.1 ml of a saline containing 1% carrageenan (Type IV, Sigma). The volume of the right hind foodpad of rats was measured with a

Table 1. Fatty acid contents (g) in 1,000 g of diets used in the present study.

Fatty acids	Diets			
	High-fat control diet	EPA diet	DHA diet	Standard chow CE-2 ¹
16:0	16.1+1.6E ²	16.1	16.1	6.9
16:1	2.4	2.4	2.4	0.6
18:0	6.4+0.6E	6.4	6.4	0.8
18:1n-9	38.6+4.4E	38.6	38.6	10.1
18:2n-6	13.4+11.8E	13.4	13.4	22.0
18:3n-3	0.8+1.6E	0.8	0.8	1.9
20:5n-3	—	19.5E	—	0.8
22:6n-3	—	—	19.5E	0.8

Fatty acids which comprised more than 0.5 g/1,000 g diet were listed. One thousand grams of the standard chow (CE-2) contained 252 g crude protein, 44 g crude fat, 44 g crude fiber, 70 g crude minerals, 502 g soluble non-nitrogen materials and 88 g water. The EPA, DHA, and high-fat control diets contained 90% lipid-free powder diet in common and 10% various lipids. Nine hundred grams of the lipid-free powder diet contained 207 g casein, 447 g corn starch, 81 g α -starch, 90 g granule sugar, 27 g cellulose powder, 9 g vitamin mixture, 36 g minerals, and 2.7 g methionine.

¹According to the manufacturer's information. ²E: provided in the form of ethyl ester.

Fig. 1. Study design of the present study. Rats entered the study when they were 5 weeks old (day 0). At the end of diets (day 15), swelling of footpads was induced by carrageenan injection into the right hind footpad. After measurement of swelling, peritoneal cells were collected. Five rats were used for each group.

volume meter (MK-550, Muromachi-Kikai, Tokyo) before the injection of carrageenan and 4 h afterward. Swelling of footpads was expressed as % original volume.

Isolation of peritoneal cells and fatty acid analysis. Right after swelling was

measured, 50 ml of Hanks' balanced salt solution was injected into the abdominal cavity under ether anesthesia. The injected fluid was recovered after mild massage of the abdomen, and peritoneal cells were collected by centrifugation. They were resuspended in Hanks' balanced salt solution and stored at -40°C until fatty acid analysis. The analysis was performed within two weeks. The fatty acid composition of the total phospholipid fraction of cells was analyzed as described previously (8). Briefly, the total lipids of peritoneal cells were extracted with chloroform/methanol (2:1, v/v). Total phospholipids were separated by thin-layer chromatography on silica gel plates. The fatty acids of phospholipids were transmethylated with 6% sulfuric acid in anhydrous methanol and were analyzed on a C-14 A gas chromatograph (Shimadzu, Kyoto) equipped with an SP-2330 capillary column (Supelco, Bellefonte, PA). The composition is expressed as mol%.

Statistics analysis. Data are expressed as $M \pm \text{SD}$. For comparison of data among the seven different groups of rats, a *t*-test was performed with Bonferroni's adjustment for six comparisons with the HF-15 (control) group after analysis of variance. To test correlationship, a least square method was performed. $p < 0.05$ was taken as significant.

RESULTS

During the experimental period, rats ate 180–200 g/group ($n=5$)/day irrespective of diets. That corresponded to 4 g EPA, DHA, or soybean oil/kg/day in the EPA, DHA, or HF groups, respectively. At the end of diets body weight of rats

Fig. 2. Body weight of rats in the seven groups at the end of diets. Seven groups of five rats were fed either of the following four diets for 5 or 15 days: the high-fat control diet (the HF groups), the EPA diet (the EPA groups), the DHA diet (the DHA groups), or standard chow (the REF group). Data are expressed as $M \pm \text{SD}$ of 4 rats. *Significantly different from the HF-15 (control) group at $p < 0.05$.

Fig. 3. The swelling of footpads 4h after injection of carrageenan in the seven groups of rats. At the end of diets the right hind footpad was injected with 1 mg carrageenan. Swelling of footpads 4h after injection was measured by a volume meter and expressed as % original volume ($M \pm SD$ of 5 rats). *Significantly different from the HF-15 (control) group at $p < 0.05$.

Table 2. Fatty acid composition of the total phospholipid fraction in peritoneal cells at the end of various diets.

Fatty acids	Dietary groups						
	HF-15 (control)	EPA-15	DHA-15	HF-5	EPA-5	DHA-5	REF
16:0	17.5 $\pm$ 1.4	18.9 $\pm$ 1.0	16.9 $\pm$ 0.9	17.3 $\pm$ 1.1	18.1 $\pm$ 1.8	17.3 $\pm$ 1.1	18.9 $\pm$ 1.8
18:0	18.7 $\pm$ 1.1	19.0 $\pm$ 1.0	18.9 $\pm$ 1.8	18.3 $\pm$ 0.7	19.3 $\pm$ 0.5	18.5 $\pm$ 1.6	19.2 $\pm$ 1.4
18:1n-9	7.5 $\pm$ 0.5	8.4 $\pm$ 0.6	8.3 $\pm$ 0.7	6.8 $\pm$ 0.6	7.8 $\pm$ 0.6	8.0 $\pm$ 0.9	6.6 $\pm$ 0.2
18:1n-7	2.8 $\pm$ 0.2	2.7 $\pm$ 0.2	2.5 $\pm$ 0.3	2.6 $\pm$ 0.3	3.0 $\pm$ 0.2	3.0 $\pm$ 0.4	2.7 $\pm$ 0.3
18:2n-6	3.9 $\pm$ 0.4	3.5 $\pm$ 0.4	4.4 $\pm$ 0.4	4.2 $\pm$ 0.6	4.1 $\pm$ 0.5	4.6 $\pm$ 0.7	6.8 $\pm$ 0.7*
20:3n-6	0.9 $\pm$ 0.1	0.8 $\pm$ 0.1	0.9 $\pm$ 0.5	1.6 $\pm$ 1.1	1.0 $\pm$ 0.2	1.3 $\pm$ 0.2	1.1 $\pm$ 0.2
20:4n-6	15.7 $\pm$ 2.8	5.1 $\pm$ 1.0*	6.2 $\pm$ 1.0*	14.0 $\pm$ 2.3	9.6 $\pm$ 1.6*	10.8 $\pm$ 2.5	13.3 $\pm$ 2.0
20:5n-3	0.03 $\pm$ 0.06	5.5 $\pm$ 0.4*	2.1 $\pm$ 0.3*	0.2 $\pm$ 0.2	4.3 $\pm$ 1.1*	1.5 $\pm$ 0.6*	0.4 $\pm$ 0.2
22:5n-3	0.8 $\pm$ 0.5	7.3 $\pm$ 0.7*	1.6 $\pm$ 0.9	2.3 $\pm$ 0.3*	6.8 $\pm$ 0.6*	2.4 $\pm$ 0.3*	2.6 $\pm$ 0.4*
22:6n-3	1.1 $\pm$ 0.1	1.1 $\pm$ 0.2	8.5 $\pm$ 0.9*	2.0 $\pm$ 0.4*	1.5 $\pm$ 0.2	6.7 $\pm$ 0.8*	2.1 $\pm$ 0.4*
24:0	1.2 $\pm$ 0.4	1.7 $\pm$ 0.7	1.9 $\pm$ 0.8	1.3 $\pm$ 0.4	1.4 $\pm$ 0.6	1.5 $\pm$ 0.7	1.2 $\pm$ 0.3
24:1n-9	2.6 $\pm$ 1.0	3.4 $\pm$ 1.4	3.1 $\pm$ 1.3	2.6 $\pm$ 0.9	2.5 $\pm$ 1.0	2.7 $\pm$ 1.5	2.0 $\pm$ 0.5

Rats were fed either the high-fat control, EPA, DHA diet (for 5 or 15 days), or the standard chow for reference (for 15 days). At the end of diets peritoneal cells were collected and analyzed for fatty acid composition of their phospholipid fraction. Data are expressed as $M \pm SD$ of 5 rats. Significant difference from the HF-15 (control) group is shown by * ($p < 0.05$).

was measured. As shown in Fig. 2, body weight of the REF and DHA-15 groups was significantly heavier than the HF-15 (control) group.

The swelling of the right hind footpad 4 h after injection of carrageenan is shown in Fig. 3. The swelling was significantly reduced by half only in the EPA-15 and DHA-15 groups compared with the HF-15 group.

The fatty acid composition of the total phospholipid fraction in peritoneal cells is shown in Table 2. Linoleic acid (LA, 18:2*n*-6) concentrations were significantly higher in the REF group than those in the HF-15 group, which reflected the higher LA composition (~50% of total fatty acids) in the standard chow (CE-2) of all the diets used in the present study. EPA was increased in the EPA groups, and DHA was increased in the DHA groups as expected. Those changes were more marked in the 15-day groups than in the 5-day groups.

We calculated the availability index of arachidonic acid (AA, 20:4*n*-6) of peritoneal cells by dividing AA concentrations by the sum of HUFA (in this case, HUFA include 20:3*n*-6, 20:4*n*-6, 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3). There was a significant positive correlation between degree of swelling and the availability index of AA among all rats at $r=0.52$ ($n=35$). If the two kinds of data are averaged within each group, the correlation coefficient would be increased to $r=0.87$ ($n=7$) as shown in Fig. 4. Deletion of data of the REF group (7 in Fig. 4), whose diet was different from the other diets in terms of fat contents and also non-fat ingredients,

Fig. 4. Correlation between the mean degree of swelling of footpads (%) and the mean availability index of AA in peritoneal cells. At the end of diets carrageenan-induced swelling was measured, and fatty acid composition of peritoneal cells was analyzed. The availability index of AA was calculated as follows: The index (mol%/mol%) = $[20:4n-6]/[20:3n-6 + 20:4n-6 + 20:5n-3 + 22:5n-3 + 22:6n-3]$. The degree of swelling and index were averaged within the same group ($n=5$ for each group). The dietary groups are indicated by numbers; 1: the HF-15 group; 2: the EPA-15 group; 3: the DHA-15 group; 4: the HF-5 group; 5: the EPA-5 group; 6: the DHA-5 group; 7: the REF group.

slightly increased the coefficient of correlation to $r=0.90$ ($n=6$). The correlation between degree of swelling and the plain AA concentrations in the total phospholipids fraction among all rats was also significant ($r=0.41$, $p<0.05$), but slightly inferior to the coefficient with the availability index of AA ($r=0.52$).

DISCUSSION

Our high-fat control diet was different from the reference diet (CE-2) in not only fat components but also the other major nutrients. Consequently, it was necessary to rule out the possibility that the control diet might have been pro-inflammatory compared with the reference diet and might have exaggerated an anti-inflammatory effect of *n*-3 HUFA. As shown in Fig. 3, there was no difference at all in inflammation severity between the control (HF-15) and reference (REF) groups.

To match the molecular form of soybean oil fatty acids with EPA and DHA ethyl esters, soybean oil was transesterified before mixed with powder diet. At the end of diets, the mean body weight of the HF-15 group, which had been on the soybean oil fatty acid-supplemented diet, was lighter than the other group and significantly different from that of the DHA-15 and REF groups. However, the difference was rather small.

The effects of *n*-3 HUFA on acute experimental inflammation induced by carrageenan have been reported from a few laboratories. Terano et al. (5) administered 240 mg/kg/day of EPA ethyl ester to rats for 4 weeks and found that carrageenan-induced edema was reduced by 20% compared with control rats. The concentrations of prostaglandin E_2 (PGE_2) and thromboxane B_2 in the inflammatory exudate induced by carrageenan-impregnated sponges were also significantly reduced in their experiment. Besides, the concentrations of leukotriene B_4 (LTB_4) in the exudate tended to be reduced. Sametz et al. (9) reported that administration of a marine oil in a dose of 50 or 100 mg EPA/kg/day for 10 weeks strongly reduced PGI_2 production but not PGE_2 production by inflamed skin, and that either dose of EPA did not influence carrageenan-induced edema. Yoshino and Ellis (10) employed a higher dose of *n*-3 HUFA, 500 mg EPA/kg/day and 333 mg DHA/kg/day, and fed rats for 50 days. They did not find any anti-inflammatory effects of *n*-3 HUFA on carrageenan-induced edema and did not find PGE_2 - or LTB_4 -reducing effects either, as far as the acute antigen-induced inflammatory exudate in the rat air-pouch model was concerned.

The results of the last two studies described above (9,10) with regard to anti-inflammatory effects of *n*-3 HUFA were different from ours. This discrepancy is probably explained by large differences in *n*-3 HUFA doses. Sametz et al. (9) employed a dose of 100 mg EPA/kg/day at most; Yoshino and Ellis (10) employed a dose of 833 mg (EPA + DHA)/kg/day. These doses were much smaller than our dose of 4 g *n*-3 HUFA/kg/day. It seems difficult to reconcile a rather low dose of EPA (240 mg/kg/day) and a positive anti-inflammatory effects of Terano et al. (5).

However, the reduction rate of inflammation by EPA administration in their study was just marginal (5).

LTB₄ and PGE₂ are very potent pro-inflammatory eicosanoids. Judging from results of Terano et al. (5), Sametz et al. (9), and Yoshino and Ellis (10), it might be concluded that beneficial effects of *n*-3 HUFA on acute inflammation is mediated through their depressive effect on LTB₄ and/or PGE₂ production. Indeed, it has been reported that a diet supplemented with 10% by weight fish oil for 3 weeks results in a 50% decrease in the amount of LTB₄ produced by rat leukocytes compared with that of control rats (11). In this context we became interested in whether changes in the fatty acid composition of inflammation-related cells induced by *n*-3 HUFA administration could explain their anti-inflammatory effects regardless of administration periods and carbon numbers of *n*-3 HUFA used. If the proportion of AA in position 2 of phospholipids is reduced by diets, the capacity for inflammatory cells to release free AA and thus synthesize eicosanoids would be reduced, too.

In the present study, we employed the availability index of AA as calculated in Fig. 4 instead of the exact proportion of AA in position 2 of phospholipids. Since HUFA are mostly located in position 2 and the HUFA other than AA may be competitive at certain steps for eicosanoid formation (1, 4, 8, 12), it is likely that the index indicates the availability of AA for eicosanoid formation. There were some other minor HUFA in position 2 besides the HUFA listed in the equation, but they were omitted from the calculation because of their minor contribution (less than 0.5% of total fatty acids). The significant correlation between the availability index of AA and degree of footpad swelling suggests the common mechanism of action of *n*-3 HUFA against acute inflammation, namely reduction in the AA availability.

Comparison of anti-inflammatory effects of EPA and DHA has not been reported yet. In the present study, their efficacy was essentially the same (Fig. 3) regardless of administration periods. Effects of EPA or DHA administration on reductions in AA availability were also similar.

Lands et al. (13) developed equations for predicting *n*-6 HUFA as % HUFA in phospholipid fractions from dietary lipid data. They applied the equations to calculating *n*-6 HUFA as % HUFA from dietary information of an independent diet-eicosanoid study of rats (14) and predicted thromboxane formation by normalization with a maximum value of thromboxane formation. Interestingly, the predicted and observed values of thromboxane in rats fitted almost perfectly (13). That indicates the utility of employing the analytically determined proportion of 20:3*n*-6 + AA in the phospholipid HUFA as a predictor of the probable intensity of *n*-6 eicosanoid formation (13). In the present study, we employed the index of AA alone and not 20:3*n*-6 plus AA because PGE₁, which is synthesized from 20:3*n*-6, might be an anti-inflammatory eicosanoid (15, 16). At any rate, the analytically determined proportion of the precursor fatty acid (AA) is also useful for predicting the modification of acute inflammatory reactions.

In conclusion EPA and DHA ameliorated acute inflammation to the same

extent, and their effects appear to be explained by the reduction of AA availability.

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