Changes in fatty acid composition in rat blood and organs after infusion of docosahexaenoic acid ethyl ester1-3

Katsuya Yamazaki, Tomohito Hamazaki, Saburo Yano, Tadashi Funada, and Fumio Ibuki

ABSTRACT An infusible emulsion of docosahexaenoic acid ethyl ester (DHA-EE) was prepared. One hundred milliliters of the emulsion contained 10 g DHA-EE (90% pure). Three milliliters of the emulsion was infused into tail veins of 22 Wistar rats weighing ~300 g. They were killed 1, 6, and 24 h and 3 and 7 d after the infusion, and fatty acid composition of various organs and plasma was analyzed along with that of control rats. DHA concentrations reached their peaks within 24 h after DHA infusion in plasma lipid fractions and in the phospholipid fraction of liver and lung. DHA did not increase at all in cardiac phospholipid fraction. However, DHA concentrations increased markedly (from 0.7% to 11%) in the free fatty acid fraction of heart 1 h after the infusion. DHA emulsion might be useful for patients in whom a rapid increment in DHA in tissues is beneficial. Am J Clin Nutr 1991;53:620-7.

KEY WORDS Docosahexaenoic acid, intravenous infusion, fatty acid composition, eicosapentaenoic acid, emulsion, free fatty acids, heart

Introduction Docosahexaenoic acid (DHA, 22:6n-3, or 22:6ω3), a major polyunsaturated fatty acid of fish oils, is found in brain, retina, sperm, and heart of land animals as a major fatty acid component in phospholipids (1). DHA is probably essential for the functional development of the nervous system (2), including the retina (3). DHA has other biomedical effects such as depression of platelet aggregation (4, 5) and augmentation of efficacy of anticancer treatment (6, 7).

Recently, McLennan et al (8) showed that feeding rats a diet supplemented with tuna fish oil, which raised the concentration of n-3 fatty acids, DHA in particular, in phospholipids of cardiac tissue, reduced the incidence and severity of arrhythmias during coronary artery occlusion and reperfusion. Swanson et al (9) reported that the increment of n-3/n-6 fatty acids induced by a fish-oil diet in the phospholipid fraction of cardiac sarcoplasmic reticulum [mostly because of DHA increase and arachidonate (AA, 20:4n-6) decrease] was associated with a lower relative activity of Ca2+-Mg2+-ATPase and a lower initial rate of calcium transport and maximum calcium uptake in sarcoplasmic reticulum vesicles. Talesnik (10) showed that a 5-min infusion of DHA into isolated perfused hearts of rats caused an inhibition of AA-induced coronary-artery constriction. Hamazaki et al (11) reported that infusion of DHA emulsion into rabbits completely prevented sudden death induced by intravenous infusion of AA, whereas death occurred in 100% of the control animals. Consequently, infusion of DHA emulsion may become a unique method to modify cardiac pathophysiology by changing DHA concentrations in cardiac tissue very quickly.

In the present study we made an infusible emulsion of DHA ethyl ester (DHA-EE) and infused it into rat tail veins to determine to what extent DHA would be incorporated in various organs. If DHA-EE infusion is clinically applied in the future to patients in whom a rapid DHA increment in tissues is beneficial, the present study would provide very basic information about doses, uptake by organs, and distribution of DHA among organs.

Materials and methods

Sardine oil was extracted from eviscerated sardines, deacidi-fied, and decolorized. After separation of saturated fatty acids by crystallization at -70 °C, a triglyceride mixture rich in polyunsaturated fatty acids was saponified and then ethylated (12). Ethyl esters of polyunsaturated fatty acids were separated to obtain a DHA-EE fraction by high-performance liquid chromatography (13). DHA-EE was emulsified with egg-yolk phospholipids according to the method of Geyer et al (14). One hundred milliliters of the DHA-EE emulsion contained 10 g DHA-EE, 1.2 g egg-yolk phospholipids, and 2.5 g glycerol for maintaining osmotic pressure. The fatty acid composition of DHA-EE and egg-yolk phospholipids used for emulsion is shown in Table 1. Twenty-six male Wistar rats weighing ~300 g each were used. For 1 wk before and throughout the infusion experiment, they were caged in groups of 4-5 under standard conditions of temperature, lighting, and water supply and fed ad libitum on a fish-oil–free diet (F-1, Funabashi Farm, Chiba, Japan). The composition of this diet and the fatty acid composition of its lipid fraction are shown in Table 1. One, 6, and 24 h and 3 and 7 d

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TABLE 1
Fatty acid composition of docosahexaenoic acid ethyl ester (DHA-EE), egg-yolk phospholipids, and fish-oil–free diet (F-1 diet) used in the experiments

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>DHA-EE</th>
<th>Egg yolk</th>
<th>F-1 diet*</th>
<th>mol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>—</td>
<td>34</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>—</td>
<td>14</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>18:1n-9</td>
<td>—</td>
<td>32</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>—</td>
<td>16</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>18:3n-3</td>
<td>—</td>
<td>—</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>20:4n-6</td>
<td>—</td>
<td>3</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>20:5n-3</td>
<td>4</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>22:6n-3</td>
<td>90</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

* Composition (wt %) was 21.5% crude protein, 4.3% crude lipids, 2.9% crude fiber, 3.9% minerals, 59.4% soluble material containing no energy, and 8.0% water.

after the infusion of 3 mL DHA-EE emulsion into tail veins of 22 rats, blood samples were collected under ether anesthesia from abdominal aortas of 4–5 rats at each time. Blood samples of the remaining four rats were also collected without the prior infusion of DHA-EE emulsion after 1 wk on the fish-oil–free diet and used for the measurement of basal concentrations (0 h). Blood was treated with the anticoagulant EDTA and centrifuged to obtain plasma. After the animals were killed by exanguinity, hearts, kidneys, livers, lungs, and spleens were excised and rinsed in saline. All samples were frozen at −80°C until the measurement of fatty acid composition. Animals were treated according to the institutions’ guidelines for the care of laboratory animals.

Serum total cholesterol (15), cholesteryl esters (CEs) (15), total phospholipids (PLs) (16), triglycerides (TGs) (17), and free fatty acids (FFAs) (18) were measured by enzymatic methods.

Total plasma lipids were extracted with 8 mL chloroform-methanol (2:1, vol:vol) from 0.4 mL plasma (19). PLs, FFAs, TGs, and CEs were separated by thin-layer chromatography (TLC) on Silica Gel 60 plates (0.25 mm, Merck, Darmstadt, FRG) with petroleum ether–diethyl ether–acetic acid (80:20:1) as a solvent system. Because of poor separation of TGs from DHA-EE on TLC, we collected TGs together with DHA-EEs.

Fatty acids of each fraction were transmethylated with 6% sulfuric acid in methanol at 70°C for 45 min. Fatty acid methyl esters were analyzed with a GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a Supelcowax-10 capillary column (30 m × 0.32 mm id, Supelco, Bellefonte, PA). The injection port was at 250°C; column temperatures were programmed at 180°C for 5 min, from 180°C to 234°C at 6°C/min, and held; detection was by flame ionization (20). Helium was used as a carrier gas with an inlet pressure of 0.56 kg/cm².

Frozen organs were thawed, minced, and homogenized three times in 8 mL chloroform-methanol (2:1) by a Polytron (Kinematica, Lucerne, Switzerland) for 10 s. The fatty acid analysis of total PL in organs was performed as for plasma lipids after the separation of total PL by TLC. For heart, the FFA fraction was also obtained by TLC and its fatty acid composition was analyzed similarly. 15:0 fatty acid (0.5 μmol/0.25 g cardiac tissue) was added before homogenization as an internal standard for the measurement of absolute FFA concentration in heart.

Data were expressed as mean ± SD. For the comparison of the data after DHA infusion with the baseline data, a paired t test was performed with Bonferroni’s adjustment after analysis of variance (ANOVA) (21). P < 0.05 was taken as significant.

Results

There were no significant changes in body weight between rats of the 0-h group and those of any other groups at the time of death. Plasma of the 1-h group was slightly turbid. Other groups had clear plasma. While collecting organs, we could not find any macroscopic abnormality or lipid accumulation in any organs. There were no significant differences in serum lipids (total cholesterol, CEs, PLs, TGs, and FFAs) among the six groups except that concentrations of PLs and FFAs were significantly increased 1.5- and 3-fold, respectively, in the 1-h group as compared with those of the 0-h group (Table 2).

Fatty acid composition of CEs, PLs, TGs, and FFAs in plasma is shown in Table 3. In the plasma CE fraction, DHA and eicosapentaenoate (EPA, 20:5n-3) reached their peaks 6–24 h after the infusion. Oleate (18:1n-9) was increased 1–6 h after the infusion. There were no other significant changes in any other fatty acids. In the plasma PL fraction, DHA reached its peak 6 h after the infusion. EPA did not show any changes. There was a marked increase in oleate 1 h after the infusion at the expense of linoleate (18:2n-6) and AA. However, in terms of

<p>| TABLE 2 |
| Changes in serum lipids after DHA infusion* |
|---------------|----------------|----------------|
| Lipids        | 0 h (n = 4)    | 1 h (n = 4)    |
|               | 6 h (n = 4)    | 24 h (n = 4)   |</p>
<table>
<thead>
<tr>
<th></th>
<th>3 d (n = 5)</th>
<th>7 d (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>1.29 ± 0.23</td>
<td>1.71 ± 0.23</td>
</tr>
<tr>
<td>CE</td>
<td>1.14 ± 0.16</td>
<td>1.09 ± 0.13</td>
</tr>
<tr>
<td>PL</td>
<td>1.55 ± 0.01</td>
<td>2.40 ± 0.21†</td>
</tr>
<tr>
<td>TG</td>
<td>1.20 ± 0.46</td>
<td>1.67 ± 0.85</td>
</tr>
<tr>
<td>FFA</td>
<td>0.66 ± 0.20</td>
<td>1.90 ± 0.38‡</td>
</tr>
</tbody>
</table>

* x ± SD. TC, total cholesterol; CE, cholesteryl ester; PL, phospholipid; TG, triglyceride; FFA, free fatty acid.
†‡Significantly different from 0 h: †P < 0.025, ‡P < 0.05.
of absolute concentration, DHA reached its peak in 1 h, and there were no significant changes between rats of the 0- and 1-h groups in linoleate and AA because of the 1.5-fold increase in plasma PL concentrations in the 1-h rats. In the plasma TG fraction, there was a marked increase in DHA in 1 h at the expense of palmitate (16:0). In the plasma FFA fraction DHA was markedly increased 1–6 h after the infusion. AA was reduced by 45% 1 h after the infusion in terms of relative concentration. However, the absolute AA concentration in the FFA fraction was increased 1 h after the infusion because of the threefold increase in plasma FFA concentrations in the 1-h rats (Table 2). Palmitate was significantly decreased 6 h after the infusion. Changes in DHA and EPA in the CE, PL, TG, and FFA plasma fractions are shown in Figure 1.

Fatty acid composition in PLs of liver, lung, and heart are shown in Table 4, Figure 2, and Figure 3, a (DHA and EPA only), respectively. In the liver PL fraction, DHA was increased significantly 1 and 6 h after the infusion. EPA reached its peak
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1 h after the infusion. Although the increase in EPA was fourfold in the 1-h as compared with the 0-h group, it did not reach statistical significance because of wide variations in the 1-h group. In the lung PL fraction, DHA reached its peak 1 h after the infusion. There were no marked changes in other fatty acids. In the cardiac PL fraction, there was no significant change in DHA among the groups. Docosapentaenoate (22:5n–3) was decreased slightly in the 24-h and 3-d groups. In the kidney PL fraction, DHA reached its peak 6 h after the infusion and EPA reached its peak 24 h after the infusion (data not shown). In the spleen PL fraction, the changes in DHA and EPA were similar to those of kidney (data not shown).

After we found that DHA did not increase at all in the cardiac PL fraction (Table 4 and Fig 3, a), we measured the fatty acid composition of the cardiac FFA fraction. When we minced hearts for the fatty acid analysis of the PL fraction, we saved about a half portion of roughly minced heart and kept it at −80 °C for possible further analysis. We separated FFAs from this saved portion. As shown in Table 5 and Figure 3, b (DHA and EPA only), DHA increased markedly 1 h after the infusion and the pattern of increment in DHA was similar to that of DHA in the plasma FFA fraction (Fig 1, d). AA was slightly increased after the infusion. In another set of similar experiments we measured the absolute FFA concentration of heart by using 15:0 fatty acid as an internal standard. The FFA concentrations of heart 0, 1, 6, and 24 h after DHA infusion were 5.8 ± 1.1 (n = 2), 11.5 ± 1.0 (n = 4), 7.6 ± 1.2 (n = 4), and 3.0 ± 0.3 (n = 4) μmol/g cardiac tissue, respectively. Animals at 3 and 7 d were not tested in this set of experiments.

Discussion

We have already reported on infusion of fish-oil emulsion into rabbits (22) and infusion of 90% pure EPA emulsion into rabbits (23) and humans (24, 25). We also reported on infusion of tridocosahexaenoyl-glycerol into rabbits (11), where we analyzed changes in the fatty acid composition of blood cells. However, the changes in fatty acid composition in organs after infusion of DHA-EE emulsion are not yet known. Recently, some investigators (8–10) reported on the importance of DHA increment in cardiac fatty acid composition with regard to cardiac pathophysiology; these reports stimulated our interest in whether the infusion of DHA-EE emulsion would increase DHA content in the cardiac PL fraction. We thought that if a single infusion of DHA-EE increased DHA content in heart, DHA-EE emulsion would have therapeutic implications for heart disease.

As shown in Table 4, DHA was not increased at all in the cardiac total PL fraction after a single infusion of DHA-EE emulsion. Rat heart had a high basal DHA content in the PL fraction (8.5%, Table 4). Consequently, any small increase in DHA by its infusion might have been hidden by the high basal DHA concentration. However, as shown in Table 5 and Figure 3, DHA was increased markedly in the cardiac FFA fraction. In view of a marked increase in DHA in the plasma FFA fraction (Table 3 and Fig 1, d), hearts probably took up free DHA from plasma. Indeed, the heart is known to take up FFA as the major energy source (26). This increment in DHA in the cardiac FFA fraction may be able to modify AA cascade in heart, because AA in the FFA fraction, and not in the PL fraction, in tissue is
first used for eicosanoid formation (27). The DHA concentrations in the FFA fraction 1 h after the infusion were actually comparable with those of AA (DHA 10.6 ± 3.6% vs AA 11.6 ± 1.4%, Table 5). The AA concentrations in the FFA fraction 6 h–7 d after the infusion were significantly elevated compared with the basal concentrations. The reason is unclear. Although this possibly harmful effect could be partially antagonized by the DHA fraction, it needs further evaluation.

The contrast between the rapid increase in free DHA in heart and the very stable fatty acid composition in the cardiac PL fraction after DHA infusion may illustrate the metabolic specificity of heart. The incorporation of DHA into the cardiac PL fraction was so slow even in the presence of abundant DHA in the FFA fraction that multiple infusion (at least three times) was necessary to obtain a significant increase in DHA content in the cardiac PL fraction (our preliminary experiments, data not shown). This contrast also illustrates how important it is to analyze the FFA fraction, especially after fatty acid infusion.

As shown in Table 2, serum concentrations of PLs were increased significantly 1 h after DHA infusion. The emulsion contained 1.2% egg-yolk PL as an emulsifier. Consequently, the increment in plasma PL shortly after the infusion was directly due to the emulsion itself. Oleate, a major fatty acid component of egg-yolk PL (Table 1), was actually markedly increased in the plasma PL fraction 1 h after the infusion (Table 3).

Serum concentrations of FFA were significantly increased 1 h after the infusion (Table 2). It is likely that a large amount of DHA-EE was hydrolyzed by lipoprotein lipase providing a large amount of free DHA to plasma. Indeed, as shown in Table 3, relative DHA concentrations in the plasma FFA fraction increased from 0.55% to 7.6% in 1 h after the DHA infusion. However, the increment in free DHA alone could not explain the threefold increase (from 0.66 ± 0.20 to 1.90 ± 0.38 mmol/L, Table 2) in total FFA concentrations. (For the increment in DHA to explain the increase, DHA content in the FFA fraction should have been 66%.) In terms of absolute FFA concentrations...
The Rf value of TG on TLC was so near that of DHA-EE that it was impossible to completely separate these two fractions on TLC. Consequently, we decided to take the TG and DHA-EE fractions together from TLC. Elevated values of DHA in the TG (with DHA-EE) fraction, especially those at 1 and 6 h after the infusion, were more or less due to the infused DHA-EE. It seems that DHA-EE was mostly cleared from plasma in 6 h because, as shown in Table 3, DHA concentrations in the plasma TG (with DHA-EE) fraction of the 6-h group (2.5 ± 1.1%) were about one-fifth of the peak value in the 1-h group (12.1 ± 6.7%). Indeed, the plasma of the 6-h group was not turbid whereas that of the 1-h group was slightly turbid.

More than 20 y ago Schlenk et al (28) showed the retroconversion of DHA to EPA in rat livers 12 h after oral administration of uniformly 14C-labeled DHA. More recently, Fischer et al (29) showed the retroconversion in humans. According to Fischer et al prostaglandin (PG) 1-M, the main urinary metabolite of PG1 (made of EPA) was excreted in the first 4 h urine collected after ingestion of DHA-EE, whereas the concentration of PG1-M was below the detection limit in the control periods. In the liver PL fraction of the present study, EPA peaked just 1 h after the infusion. This rapid retroconversion explains the detection of PG1-M by Fischer et al in 0–4 h urine samples after DHA ingestion. However, the increase in EPA concentrations in liver

in plasma, all the FFAs were increased 1 h after the infusion. This was due to increased lipase activity, which could hydrolyze TG and probably DHA-EE, or, more likely, to the extensive exchange of plasma free DHA for other FFAs in the FFA pool in organs. In the latter case, the FFA pool in organs would have been enriched in DHA shortly after the infusion as happened in hearts.

DHA, as an FFA, has been shown to act as an antagonist at the thromboxane-endoperoxide receptor and to depress platelet aggregation (5). The mean basal concentrations of free DHA in plasma (4.0 ± 1.4 μmol/L, from Tables 2 and 3) are likely to depress platelet aggregation induced by U46619 (a stable thromboxane A2 mimetic) with human platelet-rich plasma by 60% (5). The plasma concentrations of free DHA 1 h after DHA infusion (138 ± 76 μmol/L, from Tables 2 and 3) are 14 times more than the concentration that could completely inhibit platelet aggregation induced by U46619 (5). These points clearly indicate the physiological and clinical significance of an increase in plasma free DHA. The same logic could be applied to free-DHA concentrations in cardiac tissue. However, the absolute concentrations of free DHA were about one order of magnitude higher in cardiac tissue than in plasma even at the basal concentrations because of very high total FFA concentrations in heart, given that 1 g cardiac tissue is equivalent to 1 mL water.
might have been a result of a rapid exchange between free DHA and EPA in PLs of any other EPA-rich organs. We could not find macroscopic lipodosis in any organs of any rats. Consequently, it seems unlikely that particles of the DHA emulsion used in this experiment were trapped by capillary vessels. Indeed, oleate, a major fatty acid contained in the emulsifier, was not increased to any appreciable extents in liver and lung, which contained abundant capillaries (Table 4) or in kidney and spleen (data not shown). This finding contrasts with that of the plasma PL fraction of the 1-h group, where oleate was increased 3.8-fold (Table 3).

Leukotriene (LT) B₄ synthesized from AA is a powerful chemotactic agent (30). LT C₄ enhances vasopermeability and contraction of bronchiolar smooth muscle cells (30). Imbalanced production of these LTs may exacerbate inflammation, asthma, and other chronic inflammatory diseases. In in vitro experiments Lokesh et al (31) showed that DHA-enriched macrophages produced significantly smaller amounts of LTs B₄ and C₄ than did macrophages enriched in EPA, which had already been known to reduce LT B₄ production (32). However, dietary DHA may be very poorly incorporated into tissue phospholipids. Indeed, feeding rats the DHA-rich diet that contained 2% DHA (by weight) over a period of 4 wk did not increase DHA concentrations in liver and kidney PLs (33). In the present study a single infusion was enough to raise DHA concentrations in those organs. Consequently, DHA infusion may more rapidly affect patients suffering from diseases related to LT overproduction.

It is not known whether DHA infusion into animals loaded with tumors could increase DHA content in tumor cells. However, this area of investigation also has an important clinical implication; hyperthermic (6) and adriamycin (7) sensitivity in cultured L1210 murine leukemia cells and differentiation of HL-60 human leukemia cells that are due to retinoic acid and phorbol ester (34) are enhanced by enrichment of cellular fatty acids with DHA.

In conclusion, DHA-EE emulsion was safely infused into rat veins without organ lipodosis and increased DHA concentrations in the PL fraction of many organs and in the FFA fraction of heart in relatively a short time. This method of administering DHA may have clinical implications in some eicosanoid-related diseases, including heart disease and cancer. If used in animal experiments, this emulsion may reduce the feeding period necessary for accumulation of DHA in tissues and as a result facilitate the experiments.

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