

# Intravenous Infusion of Tridocosahexaenoyl-Glycerol Emulsion into Rabbits

## Effects on Leukotriene B<sub>4/5</sub> Production and Fatty Acid Composition of Plasma and Leukocytes

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### Abstract

Leukotriene (LT) B<sub>4</sub> is a major chemical activator of PMN. Inhibitory effects of oral administration of docosahexaenoic acid (DHA) on LTB<sub>4</sub> synthesis by PMN are known. We intravenously infused tridocosahexaenoyl-glycerol (DHA-TG) emulsion into rabbits in three different doses, namely 0.8, 0.4, or 0.2 g DHA/kg, and investigated the changes in LTB<sub>4/5</sub> production by ionophore-activated PMN. The averaged LTB<sub>4</sub> production by PMN was significantly reduced to 57 and 59% of baseline at 6 h after the infusion of 0.8 and 0.4 g DHA/kg, respectively ( $P < 0.05$ ), but not after the infusion of 0.2 g DHA/kg or 0.8 g soybean oil/kg. The combined concentrations of both DHA and eicosapentaenoic acid in the PMN phospholipid fraction were significantly increased at 6 h after the infusion of 0.8 or 0.4 g DHA/kg but not after the infusion of 0.2 g DHA/kg or 0.8 g soybean oil/kg. Oral administration of 0.8 g DHA/kg did not increase DHA or eicosapentaenoic acid in the PMN phospholipid fraction and did not decrease LTB<sub>4</sub> production by PMN at 6 h after administration. We suggest that the infusion of 0.4–0.8 g DHA/kg might be beneficial to patients who suffer from diseases that are related to the acute elevation of LTB<sub>4</sub> production. (*J. Clin. Invest.* 1993; 92:1253–1261.) Key words: arachidonic acid • docosahexaenoic acid • dose dependency • inflammation • phospholipids

### Introduction

Leukotriene (LT) B<sub>4</sub> is a metabolite of AA (20:4n-6) through the 5-lipoxygenase pathway (1) and a major activator of leukocytes inducing chemotaxis, aggregation, degranulation, and adhesion (2, 3). Excess production of LTB<sub>4</sub> may exacerbate pathophysiological states such as inflammation, asthma, arthritis, and psoriasis (2–4). According to recent studies, LTB<sub>4</sub> is also related to the reperfusion injury (5, 6) and cardiac arrhythmias (7) in acute myocardial infarction. Indeed, in experimental infarction induced by occlusion and reperfusion of coronary arteries, a 5-lipoxygenase inhibitor is reported to be able to reduce infarct size (6) and cardiac arrhythmias (8).

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Docosahexaenoic acid (22:6n-3, DHA),<sup>1</sup> which is a major polyunsaturated fatty acid (PUFA) of fish oil like eicosapentaenoic acid (20:5n-3, EPA), is found in retina, sperm, and heart of land animals, as the major fatty acid component in phospholipids (9). DHA is probably essential for the functional development of nervous system (10), including retina (11). DHA has some other effects such as depression of platelet aggregation (12), augmentation of the efficacy of anticancer drugs (13, 14), and prevention of arrhythmia (15).

Effects of DHA administration on LTB<sub>4</sub> synthesis were discussed in several studies (16–20). Lokesh et al. (19) reported that the LTB<sub>4</sub> and LTC<sub>4</sub> production by peritoneal macrophages of mice was reduced by dietary supplementation of a DHA-rich fish oil. However, a period as long as 10 d of oral administration of DHA might be necessary to reduce LTB<sub>4</sub> production (19). The effect of this type of intervention is not rapid enough for management of acute diseases related to LTB<sub>4</sub>.

Hamazaki et al. (21) developed tridocosahexaenoyl-glycerol (DHA-TG) emulsion previously and demonstrated that two intravenous infusions of the emulsion into rabbits completely prevented sudden death induced by intravenous injection of AA. However, the effect of the intravenous infusion of the emulsion on LTB<sub>4</sub> synthesis has not yet been investigated.

In the present study, we show that LTB<sub>4</sub> production by PMN was reduced by about one-half 6 h after the intravenous infusion of DHA-TG emulsion into rabbits.

### Methods

#### Experimental design

*Experiment 1.* Male Japanese white rabbits weighing 3.0–3.5 kg were used in the present study. They were caged separately under standard conditions of temperature, lighting, and water supply and were fed ad libitum a commercial standard rabbit diet (RM-3; Funabashi Farms, Chiba, Japan) containing soybean oil as the fat component before and throughout the experiment. A group of five rabbits was infused with DHA-TG emulsion (0.8 g DHA/kg) through ear veins over 15 min (0.8-g DHA group). Another group of five rabbits (control group) was infused with a commercial soybean oil emulsion (Intralipid; KabiVitrum AB, generously donated by Otsuka-Pharmaceuticals, Tokyo, Japan) (0.8 g soybean oil/kg) in the same manner. 10 ml of blood was obtained with a heparinized syringe from central ear arteries 1 wk before, and 6 and 24 h after the infusion of either emulsion for analysis of the LTB<sub>4/5</sub> production by PMN and fatty acid analysis of plasma. Additional heparinized blood samples were obtained 1 wk before and 24 h after the infusion for fatty acid analysis of PMN.

1. *Abbreviations used in this paper:* ANOVA, analysis of variance; DHA, docosahexaenoic acid; DHA-TG, tridocosahexaenoyl-glycerol; DTH, delayed-type hypersensitivity; EPA, eicosapentaenoic acid; PL, phospholipids; PUFA, polyunsaturated fatty acid; TG, triglycerides.

To investigate whether LTB<sub>4</sub> production by rabbit PMN was reduced by smaller doses of DHA-TG emulsion, we used two smaller doses (0.4 and 0.2 g DHA/kg) and measured LTB<sub>4/5</sub> production by PMN. Five rabbits were infused with 0.4 g/kg DHA-TG (0.4-g DHA group). Another five rabbits were infused with 0.2 g/kg DHA-TG (0.2-g DHA group). Blood samples for analysis of the LTB<sub>4</sub> production by PMN and fatty acid composition of plasma were similarly obtained 1 wk before, and 6 and 24 h after the infusion.

**Experiment 2.** To clarify the changes in the fatty acid composition of PMN at 6 h after the infusion of DHA or soybean oil, another 16 rabbits were similarly infused; 4 rabbits for the 0.8-g soybean oil (control) group and 12 rabbits for the 0.8-, 0.4-, and 0.2-g DHA groups (4 rabbits per group). Their PMN both 1 wk before and 6 h after the infusion were obtained for fatty acid analysis.

**Experiment 3.** Efficacy of the oral administration of DHA-TG emulsion was tested in another five rabbits. Since we had run out of DHA-TG emulsion at the end of experiment 2, we made a new batch of DHA-TG emulsion for this experiment. The materials and methods for preparing emulsion were exactly the same as in the case of experiments 1 and 2 (see below) except that a different batch of DHA-TG (purer than before) was used. The five rabbits were orally administered DHA-TG emulsion in a dose of 0.8 g DHA/kg through a gastric tube over 5 min without fasting as in experiments 1 and 2. Their PMN 1 wk before, and 6 and 24 h after the oral administration were obtained for LTB<sub>4/5</sub> production. Plasma was also obtained at the same time points for its fatty acid analysis. Blood samples for analysis of fatty acid composition in PMN phospholipid were also obtained at 6 and 24 h after the oral administration.

### Isolation of PMN

10 ml of blood sample obtained as described above was mixed with 10 ml of 6% (vol/vol) dextran (mol wt, 180,000; Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) in saline and allowed to sediment for 60 min at room temperature. The supernatant was applied to 10 ml of Ficoll (Pharmacia LKB Biotechnology Inc.)-Conray (Daiichi-Seiyaku, Tokyo, Japan) solution and was centrifuged at 450 *g* for 20 min at 4°C. The contaminating erythrocytes were removed by hypotonic lysis with ice-cold distilled water, and isotonicity was restored by the addition of a 1.8% NaCl solution. The suspension was again centrifuged, and the pellet was finally suspended in HBSS. The final suspension contained 0.5–1.5 × 10<sup>7</sup>/ml PMN measured by a hemocytometer. The viability was checked by a trypan blue exclusion test and always was > 95%.

### Measurement of LTB<sub>4/5</sub> production by PMN

PMN suspension (0.8 ml) was preincubated at 37°C for 10 min, and then incubated with 10 μM calcium ionophore A23187 (Sigma Chemical Co., St. Louis, MO) at 37°C for 10 min. The incubation was terminated by the addition of a half volume of ice-cold ethanol. 200 ng PGB<sub>1</sub> (Sigma Chemical Co.) was added to the incubation mixture as an internal standard. We had confirmed in a preliminary experiment that the PGB<sub>1</sub> was well separated by reverse-phase HPLC from LTB<sub>4/5</sub>. The mixture was centrifuged at 1,700 *g* for 15 min at 4°C. The supernatant was acidified with 0.1 N HCl to pH 3 and applied to a prewashed (20 ml ethanol and 20 ml water) ODS-silica minicolumn (Toyopak; Tosoh Corporation, Tokyo, Japan). The column was washed with 20 ml 15% ethanol-aqueous solution, 20 ml water, 20 ml petroleum ether, and, finally, 6 ml ethyl acetate. The ethyl acetate fraction was evaporated under a stream of nitrogen and redissolved in 100 μl ethanol. 20 μl of this sample was applied to a liquid chromatograph (model LC-6A; Shimadzu, Kyoto, Japan) equipped with an ODS column (model Shim-pack CLC-ODS, 0.46 (i.d.) × 15 cm; Shimadzu) at 30°C in a column oven (model CTO-6A; Shimadzu). The column was eluted with acetonitrile/methanol/water/acetic acid (140:80:90:0.4, vol/vol, pH 3.9) at 1.0 ml/min. The absorbance at 280 nm was monitored by an ultraviolet spectrophotometric detector (model SPD-6A; Shimadzu), and peaks were calculated with a Chromatopak recorder (model C-R3A; Shimadzu). Identification of LTB<sub>4/5</sub> was from the retention time of standard LTB<sub>4/5</sub> (Cayman Chemical Co., Inc., Ann

Arbor, MI). In preliminary experiments immunoreactivity of fractions of retention times of LTB<sub>4/5</sub> to anti-LTB<sub>4</sub> antibody (Daiichi-Kagaku, Tokyo, Japan) was confirmed. Amount of LTB<sub>4/5</sub> was determined with comparison to area of peaks of standard LTB<sub>4/5</sub> after the correction of recovery using PGB<sub>1</sub> (internal standard). The recovery of LTB<sub>4/5</sub> and PGB<sub>1</sub> from this procedure was ~ 90%.

### Preparation of DHA-TG emulsion

Sardine oil was extracted from edible parts of sardine. After separation of saturated lipids by crystallization at –70°C, a PUFA-rich triglyceride mixture was saponified and then methylated (22). Methyl esters of PUFAs were separated to obtain a DHA methyl ester fraction by HPLC (23). Free DHA, which was obtained by hydrolysis of DHA methyl ester, and glycerol were reacted, and DHA-TG was separated (21). The fatty acid composition of the DHA-TG for experiments 1 and 2 was 95% DHA, 2% EPA, 0.5% docosapentaenoic acid (22:5n-3), 0.2% oleic acid (18:1n-9), and 0.12% AA. The purity of DHA-TG for experiment 3 was 98%. DHA-TG was fortified with 0.2% α-tocopherol and emulsified with egg yolk lecithin according to Geyer et al. (24). 100 ml of DHA-TG emulsion contained 10 g DHA-TG, 1.2 g egg yolk lecithin as an emulsifier, and 2.5 g glycerol for osmotic pressure. The fatty acid composition of the egg yolk lecithin was as follows: 36% palmitic acid (16:0), 23% oleic acid, 15% stearic acid (18:0), 11% linoleic acid (18:2n-6), 4% AA, and 4% DHA. 100 ml of the commercial soybean oil emulsion contained 10 g soybean oil, 1.2 g egg yolk lecithin, and 2.5 g glycerol. The fatty acid composition of the soybean oil used was as follows: 59% linoleic acid, 22% oleic acid, 8% palmitic acid, 8% α-linolenic acid (18:3n-3), and 3% stearic acid. The fatty acid composition of the egg yolk lecithin of this emulsion was similar to that used in the DHA emulsion.

### Analysis of fatty acid composition of plasma and PMN

**Experiment 1.** Fatty acid composition of plasma was analyzed 1 wk before, and 6 and 24 h after the infusion. That of PMN was analyzed 1 wk before and 24 h after the infusion only in the 0.8-g DHA and control groups. In experiment 1, we did not measure fatty acid composition of PMN 6 h after the infusion so as not to make the animals anemic; it would have been necessary to take another 10 ml blood sample for the fatty acid measurement of PMN, and anemia might have modulated PMN population and functions. We did not measure fatty acid composition of PMN in the 0.4- and 0.2-g DHA group either, because there were no significant changes in the fatty acid composition of PMN between before and 24 h after DHA infusion even in the highest dose group.

**Experiment 2.** To obtain enough PMN and to analyze fatty acid composition of their phospholipid (PL) and FFA fractions at 6 h after the infusion of 0.8, 0.4, or 0.2 g DHA/kg, or 0.8 g soybean oil/kg, blood samples were collected before and 6 h after the infusion in a separate experiment.

**Experiment 3.** Fatty acid composition of plasma was analyzed 1 wk before, and 6 and 24 h after the oral administration of DHA-TG emulsion. Blood samples for fatty acid composition in the PMN PL fraction were not obtained before the oral administration because at this stage we were very sure that our rabbits without any treatment did not have even trace amounts of DHA or EPA at all in their PMN. Effects of anemia might also be reduced in this way.

Total lipids of plasma or PMN suspended in HBSS were extracted according to Folch et al. (25) after the addition of 1,2-dipentadecanoyl-phosphatidylcholine, tripentadecanoyl-glycerol, and free pentadecanoic acid (15:0) as internal standards for the quantitative measurement of fatty acids of respective lipid fractions. PL, TG, and FFA were separated by TLC on silica gel plates (0.25-mm Silica Gel 60; Merck, Derstadt, Germany) with petroleum ether/diethyl ether/acetic acid (80:20:1) as a solvent system for plasma samples. Fatty acids of these fractions were transmethylated with 6% H<sub>2</sub>SO<sub>4</sub> in methanol at 70°C for 45 min. Fatty acid methyl esters were analyzed with a gas chromatograph (model GC-14A; Shimadzu) equipped with an capillary column (0.32 mm i.d. × 30 m, model SP-2330; Supelco, Bellefonte, PA). The

injection port was at 250°C; column temperatures were programmed at 160°C for 10 min, from 160 to 200°C at 4°C/min, and maintained; detection was by flame ionization. Helium was used as a carrier gas with an inlet pressure of 0.56 kg/cm<sup>2</sup>.

### Statistical analysis

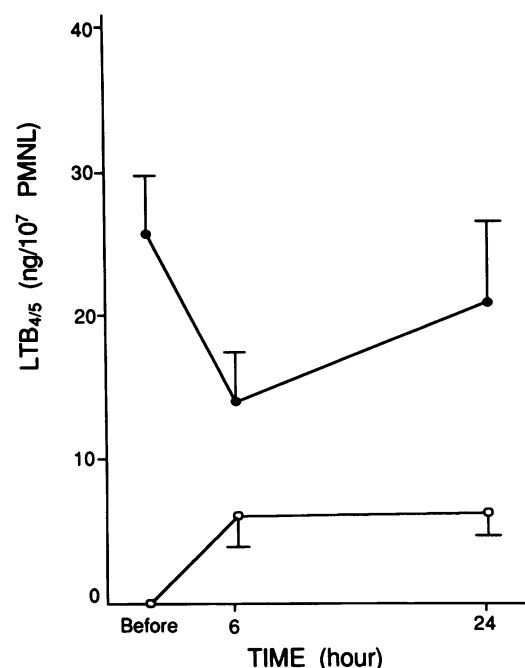
**Experiments 1 and 2.** Data are expressed as means±SD. The reduction of LTB<sub>4</sub> production by PMN after DHA infusion was analyzed by two methods. The reduction of LTB<sub>4</sub> production at 6 and 24 h was compared with baseline within the same groups by a paired *t* test with Bonferroni's adjustment after analysis of variance (ANOVA). Second, the percentage of LTB<sub>4</sub> production at 6 and 24 h compared with baseline was compared with that of the control group at the same time points by an unpaired *t* test with Bonferroni's adjustment after ANOVA. Changes in fatty acid composition were similarly compared. The comparison of LTB<sub>5</sub> production among the DHA-infused groups was performed by the unpaired *t* test with the adjustment after ANOVA. *P* < 0.05 was significant.

**Experiment 3.** Since this experiment was performed completely separately from experiments 1 and 2 with a different batch of DHA-TG emulsion, no statistical comparison between experiment 3 and experiments 1 or 2 was performed. However, changes in fatty acid composition and LTB<sub>4/5</sub> production were statistically analyzed within this experiment as described above.

## Results

### Experiment 1

**LTB<sub>4/5</sub> production by PMN.** The changes in LTB<sub>4/5</sub> production by PMN in the 0.8-g DHA group are shown in Fig. 1. The

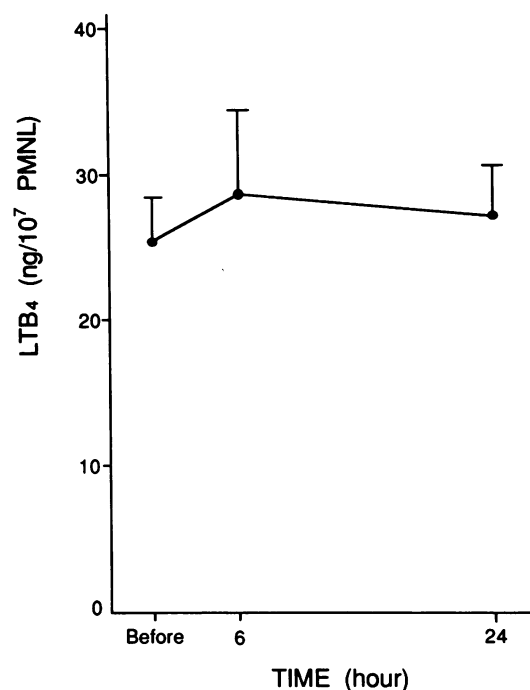


**Figure 1.** Changes in LTB<sub>4/5</sub> production by PMN after infusion of DHA-TG emulsion (0.8-g DHA group). Five rabbits were infused with 95% pure tricosahexaenoyl-glycerol (0.8 g/kg). Blood samples were obtained before, and 6 and 24 h after the infusion. PMN were prepared from them and stimulated with 10  $\mu$ M calcium ionophore A23187 for LTB production. Data are expressed as means±SD. (●) Changes in LTB<sub>4</sub> production. The production at baseline, 26.0±4.4 ng/10<sup>7</sup> PMN; at 6 h, 14.1±3.7 ng/10<sup>7</sup> PMN (57±23%, *P* < 0.05, also different from the control group at *P* < 0.025); and at 24 h, 20.9±6.6 ng/10<sup>7</sup> PMN (81±26%). (○) Changes in LTB<sub>5</sub> production. At 6 h, 5.6±2.8 ng/10<sup>7</sup> PMN; and at 24 h, 5.7±1.3 ng/10<sup>7</sup> PMN.

averaged LTB<sub>4</sub> production at 6 h after the infusion was significantly reduced to 57% of baseline value (from 26.0±4.4 to 14.1±3.7 ng/10<sup>7</sup> PMN, *P* < 0.05). This reduction was also significantly different (*P* < 0.025) from changes in the control group at 6 h (118%, see Fig. 5). The production of LTB<sub>4</sub> at 24 h after the infusion was 81% of baseline value (20.9±6.6 ng/10<sup>7</sup> PMN). At baseline, LTB<sub>5</sub> production was below the detection limits (~1 ng/10<sup>7</sup> PMN). The LTB<sub>5</sub> production at 6 h after the infusion (5.6±3.1 ng/10<sup>7</sup> PMN) was about a half amount of LTB<sub>4</sub> production at the same time point. The production at 24 h after the infusion was almost the same as that at 6 h after the infusion (5.7±3.1 ng/10<sup>7</sup> PMN).

The changes in LTB<sub>4</sub> production by PMN in the control group are shown in Fig. 2. There were no significant differences between before and after the infusion of the soybean oil emulsion in LTB<sub>4</sub> production (before, 25.2±3.9 ng/10<sup>7</sup> PMN; at 6 h after the infusion, 29.2±9.7 ng/10<sup>7</sup> PMN; and at 24 h after the infusion, 27.5±5.4 ng/10<sup>7</sup> PMN). LTB<sub>5</sub> production was below the detection limits both before and after the infusion.

The changes in LTB<sub>4/5</sub> production by PMN in the 0.4- and 0.2-g DHA groups are shown in Figs. 3 and 4, respectively. The LTB<sub>4</sub> production in the 0.4-g DHA group was significantly reduced at 6 h after the infusion (from 24.6±6.1 to 14.2±3.7 ng/10<sup>7</sup> PMN, 59%, *P* < 0.05); this reduction was also significantly different from the control group at the same time point (*P* < 0.025). There were no significant differences in LTB<sub>4</sub> production before and after the infusion in the 0.2-g DHA group (before, 25.4±8.4 ng/10<sup>7</sup> PMN; at 6 h after the infusion, 22.7±9.7 ng/10<sup>7</sup> PMN; 81%). The LTB<sub>5</sub> production after the



**Figure 2.** Changes in LTB<sub>4</sub> production by PMN after infusion of soybean oil emulsion (control group). Five rabbits were infused with soybean oil (0.8 g/kg). See the text or the legend to Fig. 1 for the experimental procedure. The production at baseline, 25.2±3.9 ng/10<sup>7</sup> PMN; at 6 h, 29.2±9.7 ng/10<sup>7</sup> PMN (118±44%); and at 24 h, 27.5±5.4 ng/10<sup>7</sup> PMN (110±24%). There were no significant changes in LTB<sub>4</sub> production before and after the infusion. LTB<sub>5</sub> production was below the detection limits before and after the infusion.

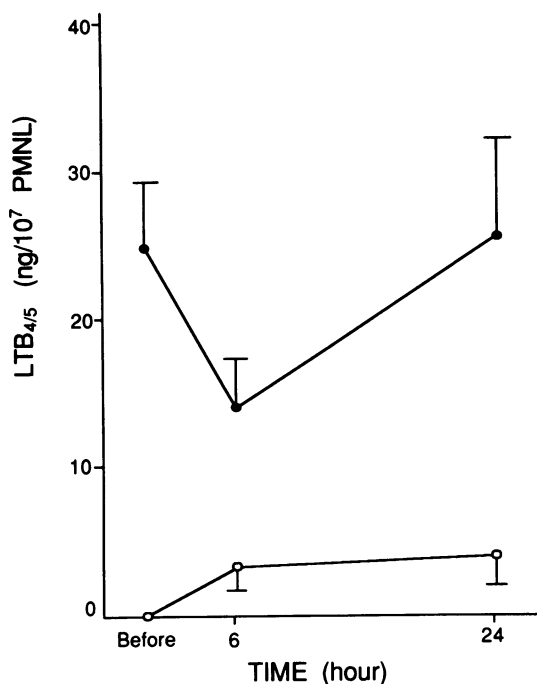


Figure 3. Changes in LTB<sub>4/5</sub> production by PMN after infusion of DHA-TG emulsion (0.4-g DHA group). Five rabbits were infused with 95% pure tridocosahexaenoyl-glycerol (0.4 g/kg). See the text or the legend to Fig. 1 for the experimental procedure. (●) Changes in LTB<sub>4</sub> production. The production at baseline, 24.6±6.1 ng/10<sup>7</sup> PMN; at 6 h, 14.2±3.7 ng/10<sup>7</sup> PMN (59±19%,  $P < 0.05$  compared with baseline value;  $P < 0.025$  compared with the control experiment at the same time point); and at 24 h, 25.5±9.1 ng/10<sup>7</sup> PMN (102±17%). (○) Changes in LTB<sub>5</sub> production. At 6 h, 3.3±1.7 ng/10<sup>7</sup> PMN; and at 24 h, 4.0±2.4 ng/10<sup>7</sup> PMN.

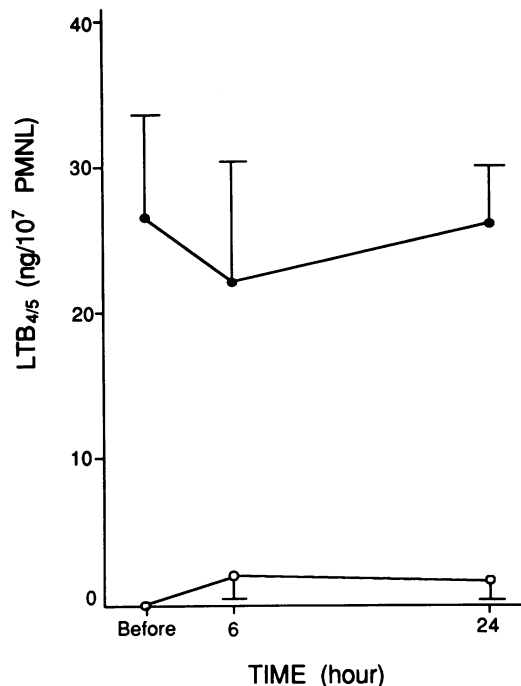


Figure 4. Changes in LTB<sub>4/5</sub> production by PMN after infusion of DHA-TG emulsion (0.2-g DHA group). Five rabbits were infused with 95% pure tridocosahexaenoyl-glycerol (0.2 g/kg). See the text or the legend to Fig. 1 for the experimental procedure. (●) Changes in LTB<sub>4</sub> production. The production at baseline, 27.4±7.0 ng/10<sup>7</sup> PMN; at 6 h, 22.7±9.7 ng/10<sup>7</sup> PMN (81±19%); and at 24 h, 26.3±4.2 ng/10<sup>7</sup> PMN (99±17%). There were no significant changes in LTB<sub>4</sub> production before and after the infusion. (○) Changes in LTB<sub>5</sub> production. At 6 h, 1.7±1.2 ng/10<sup>7</sup> PMN; and at 24 h, 1.4±0.8 ng/10<sup>7</sup> PMN.

infusion in the 0.4- or 0.2-g DHA group was detectable but smaller than that after the infusion in the 0.8-g DHA group.

The comparison of percent baseline value of LTB<sub>4</sub> production at 6 h after the infusion among the three DHA and control groups is shown in Fig. 5. The comparison of percent baseline value of LTB<sub>4</sub> production at 24 h after the infusion revealed that there were no significant differences among the three DHA and control groups (0.8-g DHA group, 81±26%; 0.4-g DHA group, 102±17%; 0.2-g DHA group, 99±17%; and control group, 110±24%). LTB<sub>5</sub> production after the infusion in the 0.8-g DHA group was significantly greater than that in the 0.2-g DHA group at either time point (at 6 h after the infusion, 5.6±2.8 vs. 1.7±1.2 ng/10<sup>7</sup> PMN,  $P < 0.025$ ; at 24 h after the infusion, 5.7±1.3 vs. 1.4±0.8 ng/10<sup>7</sup> PMN,  $P < 0.001$ ).

**Fatty acid analysis of plasma and PMN.** The changes in the plasma lipid concentrations and in the fatty acid composition of plasma lipids in the 0.8-g DHA and control groups are shown in Tables I and II, respectively. As for the 0.8-g DHA group, DHA concentrations in the plasma TG and FFA fractions were significantly increased at 6 h after DHA infusion and then decreased. DHA concentrations in the plasma PL fraction were significantly increased at 6 h after 0.8 g/kg DHA infusion and further increased at 24 h after the infusion. EPA concentrations in the plasma TG, PL, and FFA fractions were significantly increased at 6 h after the infusion, although the changes were small.

As for the control group, linoleic acid concentrations were significantly increased in the plasma TG and FFA fractions

because of a high linoleic acid concentration in the soybean oil used and were significantly decreased in the plasma PL fraction because of a low linoleic acid concentration in the egg yolk lecithin used. Oleic acid concentrations in the plasma PL and FFA fractions were also significantly increased at 6 h after the infusion of soybean oil and then decreased in the PL fraction at 24 h after the infusion. DHA concentrations in the plasma PL fraction were slightly increased at 6 h after the infusion of soybean oil and decreased at 24 h after the infusion.

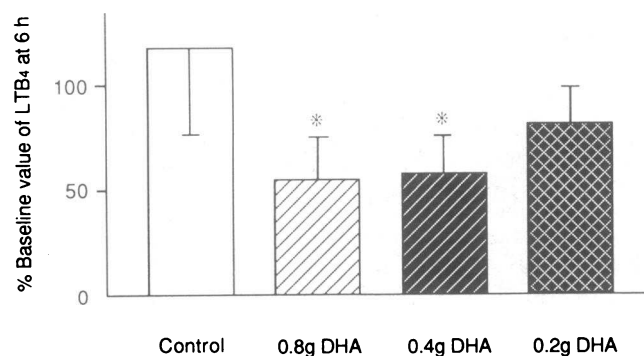


Figure 5. Comparison of percent baseline value of LTB<sub>4</sub> production in the three DHA and control groups at 6 h after the infusion. For the experimental procedure, see the text or the legends to Figs. 1–4. Data are expressed as means±SD. \* $P < 0.025$ , significantly different values from the control group.

**Table I. Changes in Plasma Lipid Concentrations and in Fatty Acid Composition of Plasma Lipids in Rabbits Infused with DHA-TG Emulsion**

	Plasma lipid fractions		
	TG	PL	FFA
<b>Lipid concentrations (mM)</b>			
0 h	0.60±0.08	0.53±0.12	0.12±0.02
6 h	1.53±1.26	0.69±0.47	0.35±0.18
24 h	1.99±0.51 <sup>‡</sup>	1.56±0.62	0.49±0.17 <sup>‡</sup>
<b>Fatty acid composition (mol%)</b>			
<b>Oleic acid</b>			
0 h	22.50±2.15	9.17±1.44	14.14±6.97
6 h	15.57±2.83 <sup>‡</sup>	11.11±1.70	15.34±3.10
24 h	20.80±2.33	8.35±0.43	16.25±1.06
<b>Linoleic acid</b>			
0 h	21.11±4.43	31.62±2.33	13.77±3.39
6 h	16.48±5.02	19.62±3.06 <sup>§</sup>	15.45±3.45
24 h	17.12±4.32	21.36±1.49 <sup>‡</sup>	14.30±3.85
<b>AA</b>			
0 h	0.14±0.03	3.09±0.12	0.59±0.61
6 h	1.08±0.27 <sup>‡</sup>	3.18±0.35	0.81±0.34
24 h	0.68±0.33 <sup>*</sup>	3.22±1.05	0.75±0.45
<b>EPA</b>			
0 h	ND	0.15±0.02	ND
6 h	1.38±0.53 <sup>‡</sup>	0.52±0.17 <sup>‡</sup>	0.55±0.21 <sup>‡</sup>
24 h	0.20±0.15	0.49±0.18 <sup>*</sup>	0.21±0.10 <sup>‡</sup>
<b>DHA</b>			
0 h	ND	0.35±0.31	ND
6 h	25.73±10.09 <sup>‡</sup>	2.44±1.10 <sup>*</sup>	5.23±2.09 <sup>‡</sup>
24 h	2.30±2.46	3.25±1.44 <sup>*</sup>	0.59±0.17 <sup>‡</sup>

Five rabbits were infused with DHA-TG emulsion (0.8 g DHA/kg). The plasma lipid concentrations and the fatty acid composition of plasma lipids at 6 and 24 h after DHA-TG infusion were compared with those before the infusion. Data are expressed as means±SD. Significantly different values from those before the infusion are shown by \*  $P < 0.05$ , <sup>‡</sup>  $P < 0.025$ , and <sup>§</sup>  $P < 0.001$ . ND, not detected.

There were no significant changes in the fatty acid composition of the total PL fraction of PMN at 24 h after the infusion in either the 0.8-g DHA or the control group (data not shown).

The changes in the plasma lipid concentrations and in the fatty acid composition of plasma in the 0.4- and 0.2-g DHA groups (data not shown) were similar to those of the 0.8-g DHA group except for the FFA fractions.

The free DHA concentrations in plasma at 6 h after the infusion in the three DHA and control groups are shown in Fig. 6. Free DHA was not detected in plasma before the infusion. The averaged concentration of plasma-free DHA was drastically increased 6 h after the infusion in the 0.8-g DHA group and then decreased thereafter (at 6 h after the infusion, 20.2±17.1  $\mu$ M; not significantly different from baseline because of wide variations; and at 24 h after the infusion, 3.1±1.5  $\mu$ M). Those at 6 h after the infusion were significantly different from the control group ( $P < 0.025$ ). The averaged concentrations of plasma-free DHA at 6 h after the infusion in the 0.4 g DHA group were slightly increased (at 6 h after the infusion,

4.6±3.4  $\mu$ M; and at 24 h after the infusion, 1.1±0.9  $\mu$ M). There was no detectable DHA in the plasma FFA fraction either before or after the infusion in the 0.2-g DHA or control group.

## Experiment 2

The fatty acid composition of PMN at 6 h after the infusion was measured in experiment 2. The DHA, EPA, and combined (DHA + EPA) concentrations in the total PL fraction of PMN at 6 h after the infusion in the three DHA and control groups are shown in Fig. 7. Neither DHA nor EPA was detected in the PL fraction of PMN before the infusion. DHA and EPA concentrations in the PL fraction of PMN at 6 h after DHA infusion were increased in a dose-dependent manner; those of the 0.8-g DHA group were significantly increased compared with baseline and also with those of the control group, but not in the other DHA groups. The combined concentrations of both DHA and EPA at 6 h after the infusion were significantly increased in the 0.8- and 0.4-g DHA groups, but not in the 0.2-g

**Table II. Changes in Plasma Lipid Concentrations and in Fatty Acid Composition of Plasma Lipids in Rabbits Infused with Soybean Oil Emulsion**

	Plasma lipid fractions		
	TG	PL	FFA
<b>Lipid concentrations (mM)</b>			
0 h	0.54±0.08	0.57±0.07	0.14±0.02
6 h	0.93±0.39	1.30±0.23 <sup>‡</sup>	0.22±0.06 <sup>*</sup>
24 h	0.92±0.28 <sup>*</sup>	0.83±0.10 <sup>‡</sup>	0.20±0.07
<b>Fatty acid composition (mol%)</b>			
<b>Oleic acid</b>			
0 h	24.57±1.74	8.75±0.97	13.38±2.26
6 h	24.44±2.15	15.27±1.66 <sup>‡</sup>	18.08±1.66 <sup>*</sup>
24 h	24.04±1.94	8.72±0.41	17.11±1.95
<b>Linoleic acid</b>			
0 h	24.46±3.94	34.46±2.26	17.44±1.82
6 h	35.69±4.54 <sup>*</sup>	26.19±2.17 <sup>‡</sup>	24.15±2.75 <sup>*</sup>
24 h	25.45±2.32	33.52±1.81	20.56±2.71
<b>AA</b>			
0 h	0.30±0.07	3.22±0.42	0.51±0.31
6 h	0.30±0.06	3.39±0.33	0.63±0.37
24 h	0.25±0.06	3.70±0.58	0.91±0.71
<b>EPA</b>			
0 h	ND	0.28±0.11	ND
6 h	ND	0.19±0.05	ND
24 h	ND	0.27±0.05	ND
<b>DHA</b>			
0 h	ND	0.26±0.17	ND
6 h	ND	1.91±0.24 <sup>‡</sup>	ND
24 h	ND	0.75±0.12 <sup>‡</sup>	ND

Five rabbits were infused with soybean oil emulsion (0.8 g soybean oil/kg). The plasma lipid concentrations and the fatty acid composition of plasma lipids at 6 and 24 h after the infusion were compared with those before the infusion. Data are expressed as means±SD. Significantly different values from those before the infusion are shown by \*  $P < 0.05$  and <sup>‡</sup>  $P < 0.025$ . ND, not detected.

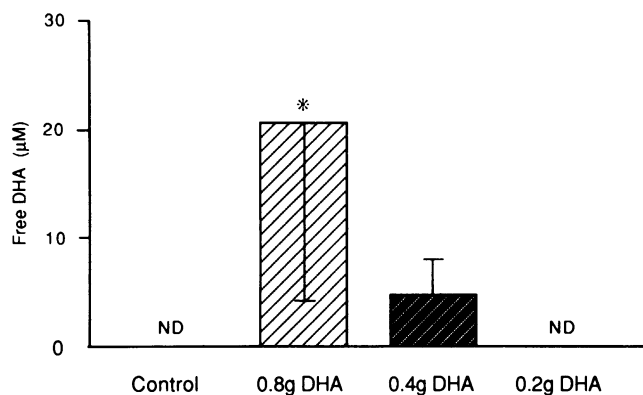


Figure 6. Comparison of free DHA concentrations in plasma at 6 h after the infusion in the three DHA and control groups. Data are expressed as means $\pm$ SD. \* $P < 0.025$ , significantly different values from the control group. ND, not detected.

DHA group, compared with baseline. The combined concentrations of the 0.8-g DHA group were significantly increased compared with the control group; those of the 0.4-g DHA group tended to be increased ( $P = 0.061$ ) compared with the control group.

The AA concentrations in the PL fraction of PMN in the three DHA and control groups are shown in Fig. 8. Those of the 0.8- and 0.4-g DHA groups were significantly reduced to  $58 \pm 10$  and  $54 \pm 8\%$  of their baseline 6 h after the infusion, respectively. Those of the 0.2-g DHA group were nonsignificantly reduced to  $65 \pm 25\%$  of baseline ( $P = 0.066$ ) 6 h after the infusion. Those of the control group were not changed before and 6 h after the infusion. The reduction rates of AA concentrations in all the three DHA groups were significantly different from the changes in AA concentrations in the control group ( $123 \pm 27\%$ ,  $P < 0.025$ ). Other major fatty acids were not significantly changed in the PL fraction of PMN at 6 h after the infusion.

Free DHA was not detected in PMN of any rabbits at 6 h after the infusion even in the 0.8-g DHA group.

### Experiment 3

**LTB<sub>4/5</sub> production by PMN.** As shown in Fig. 9, LTB<sub>4</sub> production by PMN was not significantly affected by the oral administration of DHA-TG emulsion in a dose of 0.8 g DHA/kg. LTB<sub>5</sub> production was not detected even after the oral administration.

**Fatty acid analysis of plasma and PMN.** The changes in the plasma lipid concentrations and in the fatty acid composition of plasma lipids are shown in Table III. DHA concentrations in the plasma TG and PL fractions were markedly increased but not in the FFA fraction.

Neither DHA nor EPA was detected in the total PL fraction of PMN at 6 or 24 h after the oral administration except for a very small amount of DHA (0.19%) that was detected in one rabbit at 6 h after the administration.

For reference, we compared absolute amounts ( $\mu$ M fatty acid equivalent) of DHA in plasma lipids between the intravenous and oral administration of DHA-TG emulsion in a dose of 0.8 g DHA/kg (Table IV).

## Discussion

The effects of DHA administration on in vitro (16, 18, 20) or ex vivo (17, 19) LTB<sub>4</sub> production have been discussed in several studies. However, the methods of administration of DHA in these studies were either oral or in vitro. The present study first showed the effects of intravenous administration of DHA on ex vivo LTB<sub>4</sub> production by PMN. Because the LTB<sub>4</sub> production was not significantly altered after soybean oil infusion, the reduction of LTB<sub>4</sub> production after the DHA infusion cannot be attributed to effects of infusion of a glycerol solution containing lecithin. It appears that DHA was responsible for this reduction.

There are several possible mechanisms of reduction in LTB<sub>4</sub> production by DHA infusion. (a) Increased DHA and EPA in the total PL fraction of PMN after DHA infusion probably competed with AA for phospholipase A<sub>2</sub> and/or 5-lipoxygenase, especially in the 0.8- and 0.4-g DHA groups in which the combined concentrations of both DHA and EPA were significantly increased in the total PL fraction of PMN at 6 h after the infusion. (b) LTA<sub>4</sub> hydrolase, which converted LTA<sub>4</sub> to LTB<sub>4</sub>, might be inhibited by LTA<sub>5</sub> (26), which was synthesized from EPA. As shown in Figs. 1, 3, and 4, the PMN production of LTB<sub>5</sub>, which was converted from LTA<sub>5</sub>, was produced by PMN of the DHA-infused groups in a dose-dependent manner, which suggests that reduction in LTB<sub>4</sub> production in the DHA-infused groups might be at least partly due to LTA<sub>5</sub> production. (c) The active pool of AA for LTB<sub>4</sub> production in the PL fraction of PMN might be reduced after DHA infusion. As shown in Fig. 8, the AA concentrations were significantly reduced in the PL fraction of PMN in the 0.8- and 0.4-g DHA-infused groups compared with baseline.

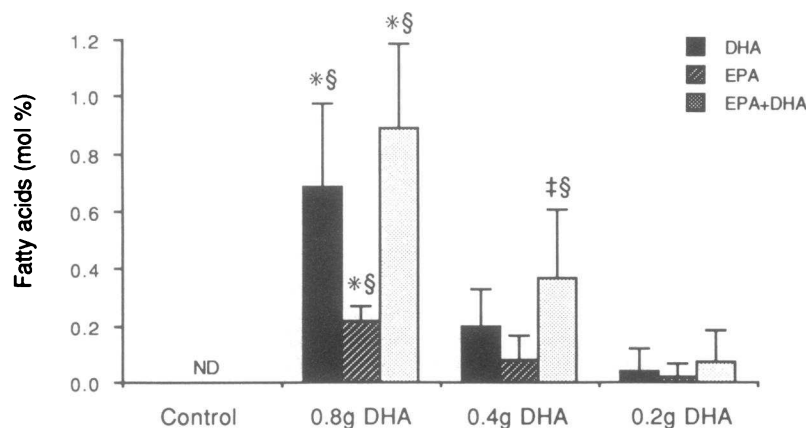


Figure 7. Comparison of DHA and EPA concentrations in the total PL fraction of PMN at 6 h after the infusion in the three DHA and control groups. Data are expressed as means $\pm$ SD. \* $P < 0.001$ ; ‡ $P = 0.061$ , significantly different values from the control group. § $P < 0.025$ , significantly increased values from baseline. ND, not detected.

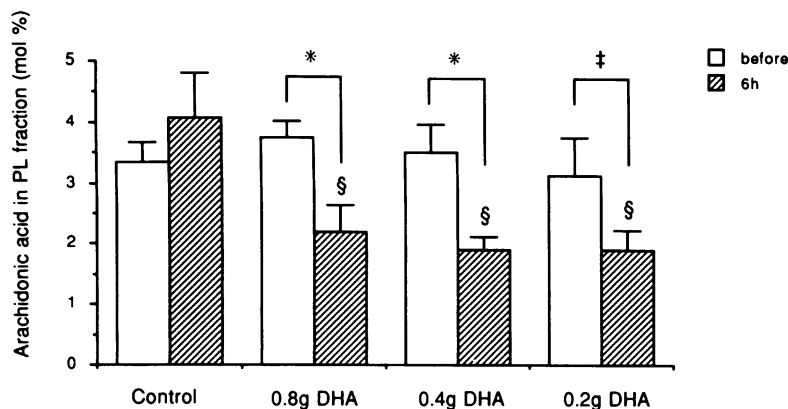


Figure 8. Comparison of AA concentrations in the total PL fraction of PMN before and 6 h after the infusion in the three DHA and control groups. Data are expressed as means $\pm$ SD. \* $P < 0.025$ ; † $P = 0.066$ , significantly different values from baseline. § $P < 0.025$ , significantly different reduction rates from the control group at 6 h after the infusion.

Before PMN were prepared for LTB<sub>4/5</sub> production analysis, they had probably been circulating for hours in free DHA-rich plasma in the 0.8- and 0.4-g DHA groups. On the other hand, we did not detect free DHA in plasma at all at 6 h after the infusion in the 0.2-g DHA or control group (Fig. 6). These findings probably account for the significant changes in DHA, EPA, and AA concentrations in PMN and, hence, changes in LTB<sub>4</sub> production in the 0.8- and 0.4-g DHA groups, but not in the 0.2-g DHA or control group. In this context, it is noteworthy that, according to Lokesh et al. (18), the *in vitro* incubation of peritoneal macrophages of mice with 15  $\mu$ M free DHA for 6

h reduced the LTB<sub>4</sub> and LTC<sub>4</sub> production from the macrophages about by half as compared with macrophages without any treatment. The concentration of free DHA used in their experiment, 15  $\mu$ M, was near the mean concentration of plasma free DHA 6 h after the infusion in the 0.8-g DHA group, namely 20  $\mu$ M.

The reason why we developed infusible DHA emulsion is that an instantaneous increase in plasma DHA concentrations by DHA infusion increases those in various tissues very quickly (27) so that DHA might rapidly take effect. However, we have not compared the two administration methods, namely oral and intravenous, by a similar experimental procedure yet. As shown in Fig. 9, the oral administration of DHA-TG emulsion did not significantly reduce LTB<sub>4</sub> production even in a dose of 0.8 g DHA/kg. As shown in Table III, the oral administration markedly increased DHA concentrations in the TG fraction at 6 h. However, the comparison of DHA concentrations in terms of millimoles of fatty acid equivalent (Table IV) indicates an unmistakable superiority of the intravenous infusion method to the oral administration, at least at 6 h after administration. The free DHA levels in plasma at 6 h after the oral administration were one-third of those of the 0.4-g DHA group at the same time point (Fig. 6). These very low free DHA levels in the oral group probably explain the failure for PMN to incorporate DHA into the PL fraction, resulting in stable LTB<sub>4</sub> production as shown in Fig. 9.

In experiments of oral administration of fish oils, which contained EPA and DHA, there was usually a reciprocal relationship between EPA, DHA, and AA (28). However, this relationship is not always the case in single-administration experiments, whether intravenous (27) or oral (29). In the present study, AA concentrations in the plasma lipid fractions were not reduced at 6 h after the administration of 0.8 g DHA/kg irrespective of the administration methods used (Tables I and III). It is not exactly clear how administered DHA maintained or even increased plasma AA concentrations. However, the presence of AA (4%) in the lecithin for emulsification may partly explain a small upward deflection of AA concentrations in plasma.

Free EPA concentrations were significantly increased at 6 h after the infusion in the 0.8-g DHA group. This increment was partly due to contaminating EPA (2%) in the DHA-TG preparation for emulsification and partly due to the retroconversion of infused DHA (30, 31). Yamazaki et al. (27) also observed an increase in EPA in the plasma cholesteryl ester fraction after DHA ethyl ester infusion in rats. In the control group, DHA

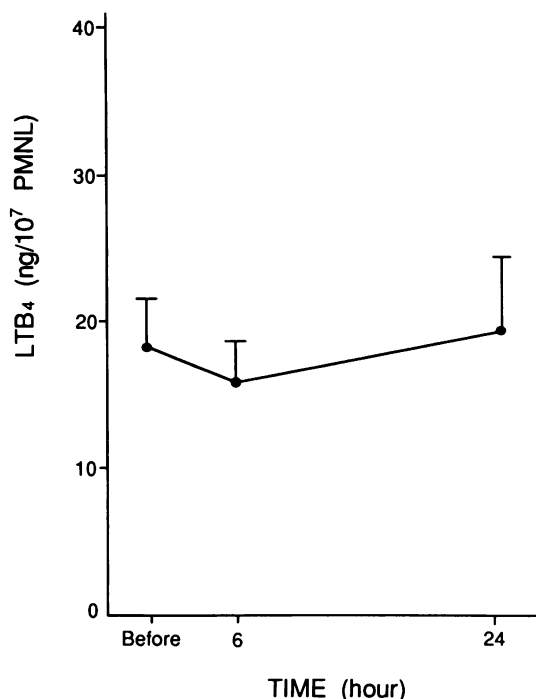


Figure 9. Changes in LTB<sub>4</sub> production by PMN after the oral administration of DHA-TG emulsion. Five rabbits were orally administered with 98% pure tridocosahexaenoyl-glycerol in a dose of 0.8 g DHA/kg. See the text or legend to Fig. 1 for the experimental procedure. (●) Changes in LTB<sub>4</sub> production. The production at baseline, 18.6 $\pm$ 2.5 ng/10<sup>7</sup> PMN; at 6 h, 16.5 $\pm$ 1.9 ng/10<sup>7</sup> PMN (88 $\pm$ 16%); and at 24 h, 19.9 $\pm$ 4.0 ng/10<sup>7</sup> PMN (107 $\pm$ 17%). There were no significant changes in LTB<sub>4</sub> production before and after the oral administration. LTB<sub>5</sub> was not detected at any time points.

Table III. Changes in Plasma Lipid Concentrations and in Fatty Acid Composition of Plasma Lipids in Rabbits Orally Administered with DHA-TG Emulsion

	Plasma lipid fractions		
	TG	PL	FFA
Lipid concentrations (mM)			
0 h	0.47±0.08	0.47±0.14	0.12±0.03
6 h	0.28±0.05	0.52±0.11	0.57±0.32
24 h	0.63±0.15	0.58±0.10	0.29±0.18
Fatty acid composition (mol%)			
Oleic acid			
0 h	26.42±5.03	8.86±1.42	13.30±6.70
6 h	20.88±2.53	8.26±0.83	21.35±2.39
24 h	19.75±1.70*	6.11±0.67†	15.92±4.94
Linoleic acid			
0 h	23.54±5.67	33.10±1.08	9.28±3.00
6 h	20.13±1.13	29.20±0.42‡	17.88±3.19§
24 h	27.73±2.78	26.05±2.74‡	16.41±3.49
AA			
0 h	0.39±0.17	3.02±0.46	ND
6 h	0.60±0.08	4.09±0.43*	0.22±0.18
24 h	0.78±0.26	3.68±0.75	0.18±0.26
EPA			
0 h	ND	0.18±0.12	ND
6 h	1.60±1.18	0.83±0.34	0.04±0.06
24 h	0.78±0.36‡	1.57±0.41‡	ND
DHA			
0 h	ND	0.46±0.07	ND
6 h	11.19±6.54*	2.38±0.32§	0.52±0.87
24 h	5.68±2.57‡	6.07±0.96§	ND

Five rabbits were orally administered with DHA-TG emulsion (0.8 g DHA/kg). The plasma lipid concentrations and the fatty acid composition of plasma lipids at 6 and 24 h after DHA-TG administration were compared with those before the administration. Data are expressed as means±SD. Significantly different values from those before the administration are shown by \*  $P < 0.05$ , †  $P < 0.025$ , and §  $P < 0.001$ . ND, not detected.

concentrations in the plasma PL fraction were slightly increased after the infusion of soybean oil. This was due to the presence of DHA (4%) in the egg yolk lecithin used.

The plasma TG concentrations of the 0.8-g DHA group at 24 h after the infusion were significantly higher than those of the control group (1.99±0.51 vs. 0.92±0.28 mM,  $P < 0.001$ ). DHA concentrations in the plasma TG fraction at 24 h after the infusion still remained slightly elevated. It seems that DHA-TG was a poor substrate for or even a competitive inhibitor against peripheral lipases and/or that hepatic uptake was slow for DHA-TG.

McLennan et al. (15) demonstrated that ventricular fibrillation induced by coronary ligation and reperfusion was able to be prevented by a long-term (10 mo) feeding on a fish oil diet that contained DHA as the major n-3 PUFA. Recently Yamazaki et al. (27) demonstrated that the infusion of DHA ethyl ester emulsion into rats increased the free DHA concentrations in plasma and heart from 0 to 8 and to 10 mol% in 1 h, respec-

Table IV. Absolute DHA Concentrations as  $\mu$ M of Fatty Acid Equivalent in Plasma Lipids

Plasma lipid fractions	Administration methods	
	Intravenous (n = 5)	Oral (n = 5)
TG		
0 h	ND	ND
6 h	1,661.0±1,922.6	73.7±55.7
24 h	194.6±215.1	100.6±44.5
PL		
0 h	4.4±5.4	4.4±1.5
6 h	43.3±35.3	25.2±8.0
24 h	141.3±99.3	70.6±14.6
FFA		
0 h	ND	ND
6 h	20.2±17.1	1.6±1.6
24 h	3.1±1.5	ND

DHA-TG emulsion was administered either intravenously (experiment 1) or orally (experiment 3) in a dose of 0.8 g DHA/kg. Since the two experiments were performed completely separately, statistical analysis was not performed. See the text or the legends to Tables I and III for the experimental procedure. ND, not detected.

tively. Taken together with these results, it is likely that this quick method of increasing the free DHA concentrations in both plasma and tissues is useful for the prevention of ventricular fibrillation if used in the very early phase of acute myocardial infarction or during pending infarction. DHA infusion may also reduce myocardial damage because it probably prevents the accumulation of PMN induced by LTB<sub>4</sub> production, which is intimately associated with the development of myocardial damage (5, 6).

In our laboratory, Taki et al. (32) observed beneficial effects of DHA-TG emulsion on delayed-type hypersensitivity (DTH) in mice. We measured the swelling of the right hindleg footpads 24 h after the injection of sheep red blood cells (SRBC) into the footpads of SRBC-immunized mice and regarded the swelling as the DTH reaction. We found that the injection of DHA-TG emulsion into tail veins just before the SRBC challenge suppressed the DTH reaction almost completely. Consequently, DHA infusion may be beneficial for the prevention of acute allograft rejection, which is similar to the DTH reaction.

In addition, more recently we observed that acute inflammation in rat footpads induced by carrageenin was depressed by the injection of DHA-TG emulsion into rat tail veins just before the carrageenin injection into footpads and that the injection of DHA-TG emulsion was as effective as the injection of 225 mg/kg of aspirin DL-lysine (unpublished data). These beneficial effects of DHA emulsion, including what is described in the previous paragraph, are probably explained partly by its effect on LTB<sub>4</sub> production.

The intravenous infusion of DHA into rabbits increased the DHA and EPA concentrations of plasma and PMN, reduced the AA concentrations of PMN, and thus significantly reduced LTB<sub>4</sub> production by PMN by about one-half 6 h afterward. However, a single oral administration of DHA did not increase the DHA or EPA concentrations of PMN and, consequently, did not modify LTB<sub>4</sub> production by PMN. These re-



sults suggest that the intravenous infusion of DHA-TG emulsion might be beneficial to patients who suffer from inflammatory diseases that are related to the acute elevation of LTB<sub>4</sub> production.

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