Low Doses of Eicosapentaenoic Acid, Docosahexaenoic Acid, and Hypolipidemic Eicosapentaenoic Acid Derivatives Have No Effect on Lipid Peroxidation in Plasma

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ABSTRACT: It was of interest to investigate the influence of both high doses of eicosapentaenoic acid (EPA) and low doses of 2- or 3-methylated EPA on the antioxidant status, as they all cause hypolipidemia, but the dose required is quite different. We fed low doses (250 mg/d/kg body wt) of different EPA derivatives or high doses (1500 mg/d/kg body wt) of EPA and DHA to rats for 5 and 7 d, respectively. The most potent hypolipidemic EPA derivative, 2,2-dimethyl-EPA, did not change the malondialdehyde content in liver or plasma. Plasma vitamin E decreased only after supplementation of those EPA derivatives that caused the greatest increase in the fatty acyl-CoA oxidase activity. Fatty acyl-CoA oxidase activity increased after administration of both EPA and DHA at high doses. High doses of EPA and DHA decreased plasma vitamin E content, whereas only DHA elevated lipid peroxidation. In liver, however, both EPA and DHA increased lipid peroxidation, but the hepatic level of vitamin E was unchanged. The glutathione-requiring enzymes and the glutathione level were unaffected, and no significant changes in the activities of xanthine oxidase and superoxide dismutase were observed in either low- or high-dose experiments. In conclusion, increased peroxisomal β-oxidation in combination with high amounts of polyunsaturated fatty acids caused elevated lipid peroxidation. At low doses of polyunsaturated fatty acids, lipid peroxidation was unchanged, in spite of increased peroxisomal β-oxidation, indicating that polyunsaturation is the most important factor for lipid peroxidation.

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Administration of peroxisome-proliferating hypolipidemic compounds produces marked increases in the enzyme activities of the peroxisomal fatty acid β-oxidation cycle, including the hydrogen peroxide-generating enzyme fatty acyl-CoA oxidase (FAO, E.C. 1.3.3.6) (1). In contrast, hydrogen peroxide-degrading enzymes such as catalase (E.C. 1.11.1.6) are not proportionally induced by peroxisome proliferators (1). This imbalance between hydrogen peroxide-generating and -degrading enzymes is an undesirable effect of the peroxisome-proliferating compounds as it may create cellular oxidative stress.

Membrane fatty acid composition can be rapidly modified by changing the source of dietary lipids (2). When polyunsaturated oils are increased in the diet, tissues become enriched with polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Membranes containing increased levels of these fatty acids are more easily oxidized (3), and the susceptibility of liver lipids to peroxidation is increased after ingestion of diets high in fish oil (4). Increasing polyunsaturation has been reported to be antagonistic to vitamin E status (5). A fish oil-containing diet may thus have an unfavorable effect on the antioxidant/prooxidant balance. In contrast, we showed that hypolipidemic doses of EPA enhance the hepatic antioxidant defense (6). PUFA have a hypolipidemic effect when administered to rats. However, high doses of EPA are necessary to cause hypolipidemia, whereas high doses of DHA have no lipid-lowering effect (7). The hypolipidemic effect of EPA was potentiated by branching, and the resulting 2- and 3-methylated EPA molecules caused hypolipidemic effects at low doses (Vaagenes, H., Madsen, L., Dyrøy, E., Elholm, M., Stray-Pedersen, A., Frøyland, L., Lie, Ø., and Berge, R.K., submitted for publication). It was of interest to investigate the influence of the above-mentioned fatty acids on the antioxidant status in rats, as they all cause hypolipidemic effect, but the doses of PUFA required to achieve this effect are different. For this purpose we measured the content of vitamins E, A and C, thiols, and lipid peroxidation, in addition to the enzyme activities of several enzymes participating in the antioxidant defense system.

MATERIALS AND METHODS

Chemicals. Ethyl esters of EPA (97.0% pure), DHA (91.2% pure), and the ethyl esters of the EPA derivatives 2-methyl-EPA (93.8% pure), 2-ethyl-EPA (96.1% pure), 2,2-dimethyl-EPA (95.3% pure), and 3-methyl-EPA (>90% pure) were obtained from Norsk Hydro AS, Research Centre (Porsgrunn, Norway). [α-^{32}P]deoxycytidine triphosphate (3000 Ci/mmol) was from Amersham (Buckinghamshire, England). Restriction enzymes were from Promega (Madison, WI). Nylon membranes (NY 13N) were from Schleicher & Schuell (Das-

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAO, fatty acyl-CoA oxidase; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; SOD, superoxide dismutase; XDH, xanthine dehydrogenase; XOX, xanthine oxidase.

sel, Germany). All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals and treatments. Male Wistar rats from Möllegaard breeding Laboratory (Ejby, Denmark), weighing 160–180 g, were housed as pairs in plastic cages in a room maintained at 12 h light-dark cycles and a constant temperature of 20 ± 3°C. The animals were acclimatized for at least 1 wk under these conditions before the start of the experiment. The different fatty acids (all as ethyl esters) were suspended in 0.1% carboxymethylcellulose, and 0.5% (wt/vol) α-tocopherol acetate was added to the PUFA to prevent autooxidation. In the low-dose experiment EPA, DHA, and the EPA derivatives were administered at a dose of 250 mg/d/kg body wt for 5 d by gastric intubation in a final volume of 0.5 mL once a day. In the high-dose experiment, EPA, DHA, and oleic acid were administered at a dose of 1500 mg/d/kg body wt for 7 d by gastric intubation in a final volume of 1.0 mL once a day. In both experiments the control animals received only sodium carboxymethylcellulose. All animals had free access to water and food (rat and mouse standard diet, from B&K Universal, Sollentuna, Sweden). The food contained 61.0 mg/kg DL-α-tocopherol acetate and 7.0 mg/kg retinol. At the end of the feeding period, after overnight fasting, the animals were anesthetized by Hypnorm (Janssen Pharmaceutical Ltd., Oxford, England) Dormicum® (F. Hoffmann-La Roche AG, Basel, Switzerland) (Fentanyl/fluanisone-Midazolam), 0.2 mL/100 g body wt. Cardiac puncture was performed to obtain blood samples, and the liver was removed. Parts of the liver were immediately frozen in liquid nitrogen, and the rest of the liver was chilled on ice for homogenization.

Preparation of subcellular fractions. The livers were homogenized in ice-cold sucrose solution (0.25 M sucrose in 10 mM HEPES buffer pH 7.4 and 1 mM EDTA) using a Potter-Elvehjem homogenizer. The subcellular fractions were isolated as previously described (8). Briefly, the homogenate was centrifuged at 1,000 × *g* for 10 min to separate the postnuclear from the nuclear fraction. A mitochondrial-enriched fraction was prepared from the postnuclear fraction at 10,000 × *g* for 10 min. A peroxisome-enriched fraction was prepared by centrifugation of the postmitochondrial fraction at 23,500 × *g* for 30 min. A microsomal-enriched fraction was isolated from the postperoxisomal fraction at 100,000 × *g* for 1 h 15 min. The remaining supernatant was collected as the cytosolic fraction. The procedure was performed at 0–4°C, and the fractions were stored at −80°C. Protein was assayed using the BioRad protein assay kit (BioRad, Richmond, CA) using bovine serum albumin as a standard.

*Vitamins E and A***.** Both liver and plasma were analyzed for vitamin E (α-tocopherol) and vitamin A (retinol) by high-performance liquid chromatography (HPLC). Plasma (200 μL) was mixed with an equal volume of 300 μM standard (α-tocopherol acetate) in ethanol, or 200 μL 10% (wt/vol) liver homogenate with 300 μM standard was added to an equal volume of water. To these solutions 500 μL hexane was added. The resultant mixture was shaken vigorously for 2 min, centrifuged, and partly evaporated (250 μL) under a stream of N_2. Then 125 μL ethanol was added, and the mixture was analyzed by HPLC. The analysis was performed with Supelcosil LC-8 column (25 cm × 4.6 mm i.d.) with LC-8 guard column packed with Perisorb RP-8 (2 cm × 2 mm i.d.). Methanol/H_2O, 95:5, and methanol were used as mobile phases. The flow was set to 1.0 mL/min and the injection volume was 40 μL. Detection was achieved at 292 nm using a Shimadzu RF-535 detector with multiplicator (Hamamatsu R928-08).

Vitamin C. Vitamin C (ascorbic acid) was measured in liver by HPLC. Two hundred microliters 10% (wt/vol) liver homogenate in 50 mM perchloric acid was added to 400 μL deproteinization solution [7% (vol/vol) perchloric acid, 1% (vol/vol) metaphosphoric acid], shaken vigorously for 20 s, frozen at −80°C, and centrifuged at 12,000 × *g* for 5 min. Supernatant was added 1:1 to the mobile phase [20 mM $NH_4H_2PO_4$, 0.015% (vol/vol) metaphosphoric acid, pH 3.0], and 20 μL was injected onto a Supelcosil LC-8 column (25 cm × 4.6 mm i.d.) with LC-8 guard column packed with Perisorb RP-8 (2 cm × 2 mm i.d.). Detection was achieved at 245 nm using a Spectra FOCUS detector.

Malondialdehyde (MDA). MDA was measured in plasma and liver by HPLC. As stated by Halliwell and Chirico (9), the HPLC-based MDA test can be used as a preliminary general measurement of lipid peroxidation. Briefly, 250 μL 20% (wt/vol) acetic acid and 250 μL 0.8% (wt/vol) 2-thiobarbituric acid in 0.1 N NaOH was added to 100 μL liver homogenate [10% (wt/vol) in sucrose solution (0.25 M sucrose in 10 mM HEPES buffer pH 7.4 and 1 mM EDTA)] or plasma. The sample was then heated at 95°C for 1 h and then chilled, an equal volume of 1-butanol was added, and the resultant mixture was shaken vigorously for 2 min. After centrifugation at 1,700 × *g* for 5 min, the supernatant was analyzed by HPLC. The analysis was performed with Supelcosil LC-18 column (5 μm, 25 cm × 4.6 mm i.d.). KH_2PO_4 (25 mM, pH 7)/methanol, 65:35, was used as mobile phase. The flow was set to 1.0 mL/min, and the injection volume was 20 μL. Detection was achieved using a Shimadzu RF-535 detector with multiplicator (Hamamatsu R928-08), with excitation at 532 and emission at 553 nm.

Enzyme activities. Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was measured spectrophotometrically by monitoring the decay of superoxide ($O_2^{\bullet -}$) (10). FAO activity was determined in the peroxisome-enriched fraction of rat livers by the coupled assay (11). The production of hydrogen peroxide was measured by monitoring the increase in dichlorofluorescein absorbance in the presence of palmitoyl-CoA.

Preparation of hybridization probes. DNA fragments were labeled by random priming using the oligolabeling technique of Feinberg and Vogelstein (12). The DNA probes were purified fragments of cloned rat genes. Copper zinc (CuZn)-SOD: 600 bp *Eco*RI fragment of pCuSOD (13), and manganese (Mn)-SOD: 1400 bp *Eco*RI fragment of pMnSOD (13). As control we used rat P0 rRNA: 1046 bp *Bam*HI/*Xho*I fragment in pBluescript II SK (z29530, provided by A. Molven, University of Bergen, Norway) or human 28S rRNA: 1400 bp *Bam*HI fragment of pA (Gonzalez, I.L., personal communication).

RNA purification and analysis. Total cellular RNA was isolated by the guanidinium-thiocyanate method described by

Chomczynski and Sacchi (14). The RNA concentrations were determined spectrophotometrically. RNA was blotted onto nylon (15). Hybridization to immobilized RNA was performed in the presence of 50% formamide, 5 × saline-sodium citrate, 200 μg/mL heat-denatured herring sperm DNA, 0.1% (wt/vol) sodium dodecyl sulfate, 25 mM sodium phosphate pH 6.5, 8.25% dextran sulfate at 42°C for 24 to 48 h (16). Filters were washed to high stringency [0.2 × saline-sodium citrate, 0.1% (wt/vol) sodium pyrophosphate, 0.1% (wt/vol) sodium dodecyl sulfate at 65°C] and Kodak XAR-5 X-ray films were exposed in the presence of intensifying screens at −80°C. Densitometric scanning of autoradiograms was performed using the LKB Ultrogel laser–densitometer (Bromma, Sweden). When the filters were to be rehybridized, the bound probe was first stripped off in 0.1% (wt/vol) sodium dodecyl sulfate at 90–100°C for 7 min. The hybridization results were normalized to the signal of a cDNA probe for a ribosomal protein used as control or 28S rRNA hybridization in the individual samples. Relative mRNA inductions of the different genes were then calculated.

Thiols. Glutathione, cysteine, cysteinylglycine, and homocysteine were quantified in plasma and liver after derivatization with the fluorescent agent monobromobimane by reversed-phase HPLC (17).

Presentation of results. The results are reported as means ± SD from 3 to 8 animals. Statistical analysis was by one-way analysis of variance.

RESULTS

Effects on vitamins. Table 1 shows the changes in vitamin E, vitamin A and vitamin C in the different dietary groups. At low doses the plasma vitamin E content decreased 35 and 22%, respectively, in rats fed 2,2-dimethyl- and 3-methyl-EPA compared to EPA, while the plasma level of vitamin A was unchanged. In liver, the vitamin E content was unchanged, while vitamin A increased in rats fed EPA. At high doses, plasma vitamin E decreased 43% in rats fed EPA and DHA, but the hepatic content of vitamin E was unchanged. Plasma vitamin A, hepatic vitamin A and hepatic vitamin C were unaffected by the different feeding groups of the high-dose experiment (Table 1).

Effect on MDA. MDA, a product of lipid peroxidation, was unchanged in plasma and liver of rats fed a low dose of EPA derivatives, EPA, or DHA, compared to controls (Table 2). In the high-dose experiment, DHA feeding caused a 1.5- and 1.6-fold elevation of plasma and hepatic MDA, respectively. EPA-feeding at high doses did not affect the plasma MDA, but the hepatic MDA increased 1.4-fold (Table 2).

Effect on plasma and hepatic thiols. When measuring the plasma levels of total homocysteine, cysteine (the rate-limiting precursor amino acid in glutathione synthesis), glutathione, and cysteinylglycine (the breakdown product of glutathione), we found that low doses of 3-methyl-EPA caused a small increase in homocysteine, while glutathione, cysteine and cysteinylglycine were unchanged (Table 3). In liver, the cysteine and glutathione levels were unchanged (Table 3). In the high-dose experiment, we found no significant changes of the hepatic thiols after treatment with EPA, DHA, or oleic acid compared to control (data not shown).

Effect on enzyme activities and mRNA levels. In the low-dose experiment 2,2-dimethyl-EPA tended to increase total SOD activity in the peroxisome-enriched fraction, although not significantly (Table 4). This was accompanied by a 1.9-fold elevated mRNA level of Mn-SOD compared to the control or EPA, and a small (1.3-fold) elevation of CuZn-SOD mRNA compared to EPA. At high doses, the enzyme activity

TABLE 1
Effects of Different Doses of n-3 Fatty Acids on Plasma and Hepatic Levels of Vitamins E and A, and Hepatic Level of Vitamin C[a]

	Vitamin E		Vitamin A		Vitamin C
	Plasma (μmol/L)	Tissue (nmol/g liver)	Plasma (μmol/L)	Tissue (nmol/g liver)	Tissue (μmoL/g liver)
Low-dose experiment (250 mg/d/kg body wt)					
2-Methyl-EPA	12.3 ± 1.7	110.2 ± 20.8	2.3 ± 0.5	25.7 ± 5.1	n.d.
2-Ethyl-EPA	11.2 ± 3.0	103.9 ± 14.2	2.4 ± 0.7	26.1 ± 7.3	n.d.
2,2-Dimethyl-EPA	9.0 ± 0.3[b]	100.7 ± 8.3	1.8 ± 0.8	23.6 ± 5.9	n.d.
3-Methyl-EPA	7.6 ± 0.3[b,c]	100.7 ± 10.5	2.5 ± 0.6	24.8 ± 2.8	n.d.
EPA	11.6 ± 1.0	112.1 ± 9.3	2.4 ± 0.3	33.0 ± 4.4[c]	n.d.
DHA	12.1 ± 1.8	101.5 ± 6.6	2.5 ± 0.8	25.3 ± 5.6	n.d.
Control	10.9 ± 1.0	110.1 ± 11.6	2.3 ± 0.5	22.4 ± 3.7[b]	n.d.
High-dose experiment (1500 mg/d/kg body wt)					
EPA	8.8 ± 1.2[c]	81.5 ± 15.8	2.1 ± 0.0	13.6 ± 1.8	1.4 ± 0.1
DHA	8.7 ± 1.4[c]	73.7 ± 7.4	2.2 ± 0.3	13.1 ± 1.9	1.6 ± 0.1
Oleic acid	13.2 ± 1.7[b]	70.1 ± 25.1	2.2 ± 0.3	13.0 ± 3.4	1.7 ± 0.3
Control	15.3 ± 1.1[b]	80.7 ± 5.4	2.1 ± 0.2	14.0 ± 2.6	1.6 ± 0.3

[a]The values are means ± SD for 4–8 animals. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; n.d., not determined.
[b]Significantly different from control (sodium carboxymethylcellulose).
[c]Significantly different from EPA ($P < 0.05$).

TABLE 2
Plasma and Hepatic Levels of MDA in Rats Fed Different Fatty Acids at Different Doses[a]

	Plasma (μmol MDA/L)	Tissue (nmol MDA/g liver)
Low-dose experiment (250 mg/d/kg body wt)		
2,2-Dimethyl-EPA	1.6 ± 0.1	44.8 ± 3.1
3-Methyl-EPA	1.6 ± 0.2	45.3 ± 6.0
EPA	1.5 ± 0.2	40.4 ± 0.8
DHA	1.4 ± 0.1	43.6 ± 3.2
Control	1.4 ± 0.1	43.6 ± 4.4
High-dose experiment (1500 mg/d/kg body wt)		
EPA	1.5 ± 0.3	112.5 ± 9.1[b]
DHA	1.9 ± 0.2[b]	127.7 ± 15.0[b]
Oleic acid	1.3 ± 0.1	84.1 ± 4.2[c]
Control	1.3 ± 0.1	81.2 ± 4.4[c]

[a]Values are means ± SD for 3–7 animals.
[b]Significantly different from control.
[c]Significantly difference from EPA ($P < 0.05$). MDA, malondialdehyde; for other abbreviations see Table 1.

of total SOD was unchanged in rats fed EPA and DHA. However, the SOD activity tended to decrease in rats fed oleic acid and there was a reduced expression of CuZn-SOD and Mn-SOD mRNA by oleic acid (Table 4). The SOD activity was unchanged in the mitochondrial-enriched and the cytosolic fractions in both the high- and low-dose experiments (data not shown). Low doses of 2,2-dimethyl- and 3-methyl-EPA or high doses of EPA and DHA did not affect the enzyme activities of xanthine oxidase (XOX, E.C. 1.1.3.22) and xanthine dehydrogenase (XDH, E.C. 1.1.1.204) compared to the control in the postnuclear or the cytosolic fractions (data not shown). Also, the activities of glutathione reductase (E.C. 1.6.4.2), glutathione peroxidase (E.C. 1.11.1.9), and glutathione-*S*-transferase (E.C. 2.5.1.18) were unchanged in the postnuclear, cytosolic or peroxisomal fractions (data not shown), as was the mRNA level of glutathione peroxidase (data not shown) in both experiments.

The enzyme activity of FAO was measured both in the low-dose (Fig. 1A) and the high-dose (Fig. 1B) experiments. Low doses of 2,2-dimethyl- and 3-methyl-EPA increased the FAO activity 2.7- and 2.2-fold, respectively, whereas 2-ethyl-EPA increased the FAO activity 1.7-fold compared to control.

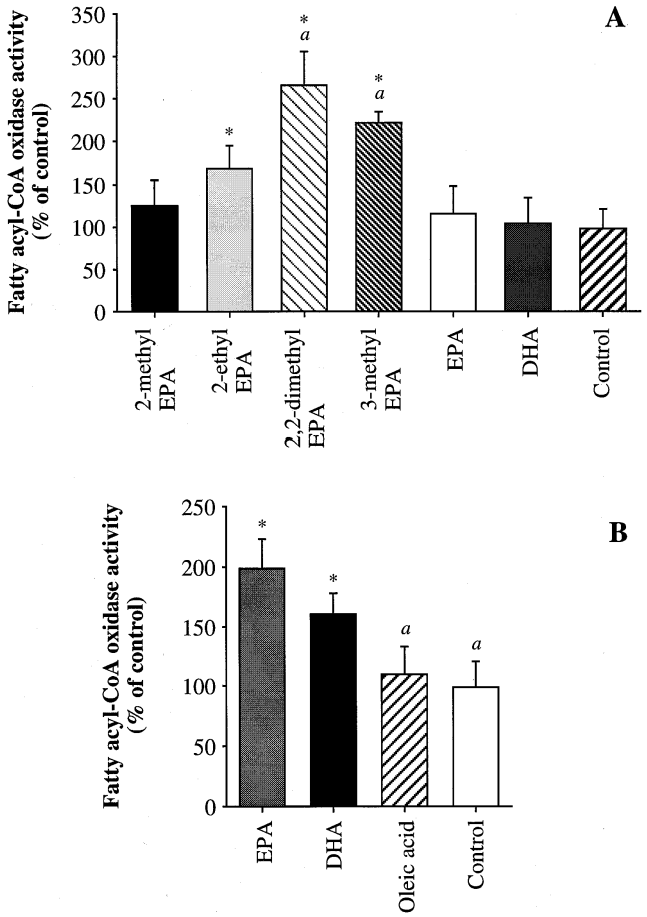

FIG. 1. Enzyme activity of fatty acyl-CoA oxidase in rats fed (A) low doses (250 mg/d/kg body wt) of the EPA derivatives, EPA or DHA, and (B) high doses (1500 mg/d/kg body wt) of EPA, DHA, or oleic acid. CMC-fed animals were used as control. Fatty acyl-CoA oxidase activity was measured in the peroxisome-enriched fraction. Data are relative percentage change from the means of the control values of 20.05 ± 4.08 and 16.41 ± 3.46 nmol/min/mg protein for (A) and (B), respectively. Data are given as means ± SD for 4–5 rats. *Significantly different from control. [a]Significantly different from EPA ($P < 0.05$). Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; CMC, sodium carboxymethylcellulose.

In the high-dose experiment EPA and DHA increased the FAO activity 2.0- and 1.6-fold, respectively.

TABLE 3
Plasma and Hepatic Thiols in Rats Fed Different n-3 Fatty Acids at Low Doses[a]

	Cysteine		Glutathione		Cys-Gly	Homocysteine
	Plasma (μmol/L)	Liver (nmol/g)	Plasma (μmol/L)	Liver (μmol/g)	Plasma (μmol/L)	Plasma (μmol/L)
2,2-Dimethyl-EPA	58.8 ± 9.1	100.1 ± 3.3	13.2 ± 1.7	4.5 ± 1.1	0.9 ± 0.1	5.6 ± 0.6
3-Methyl-EPA	71.0 ± 5.4	108.9 ± 0.6	15.1 ± 5.4	5.0 ± 1.0	0.9 ± 0.0	6.3 ± 0.3[b]
EPA	64.7 ± 4.5	108.1 ± 4.9	12.2 ± 1.8	4.7 ± 1.5	0.8 ± 0.1	5.6 ± 0.6
DHA	63.8 ± 6.9	115.6 ± 2.8	12.1 ± 2.2	5.9 ± 0.6	1.0 ± 0.1	4.9 ± 0.8
Control	62.6 ± 13.8	107.2 ± 3.7	11.8 ± 0.9	5.3 ± 1.2	1.0 ± 0.1	4.8 ± 0.5

[a]Values are means ± SD for 3–6 animals. EPA, DHA, and EPA derivatives were fed at a dose of 250 mg/d/kg body weight for 5 d.
[b]Significantly different from control ($P < 0.05$). Cys-Gly, cysteinylglycine; for other abbreviations see Table 1.

TABLE 4
Enzyme Activity and mRNA Level of Superoxide Dismutase (SOD) in Liver of Rats Fed n-3 Fatty Acids and EPA Derivatives[a]

	Total SOD activity (U/mg)	mRNA expression (relative levels)	
		CuZn SOD	Mn SOD
Low-dose experiment (250 mg/d/kg body wt)			
2,2-dimethyl EPA	1006 ± 481	1.3 ± 0.4[b]	1.9 ± 0.5[b,c]
3-Methyl-EPA	594 ± 74	0.8 ± 0.3	1.3 ± 0.3
EPA	671 ± 116	0.6 ± 0.2	1.0 ± 0.3
Control	684 ± 147	1.0 ± 0.4	1.0 ± 0.1
High-dose experiment (1500 mg/d/kg body wt)			
EPA	407.0 ± 74.9	0.7 ± 0.1	1.0 ± 0.2
DHA	378.3 ± 32.8	0.9 ± 0.2	1.2 ± 0.3
Oleic acid	370.7 ± 8.5	0.9 ± 0.1	1.0 ± 0.2
Control	460.3 ± 62.3	1.0 ± 0.3	1.0 ± 0.2

[a]The enzyme activity of SOD was measured in the peroxisomal-enriched fraction. RNA purification and hybridization experiments were performed as described in the Materials and Methods section, and the mean for the control from the mRNA data is set to 1. The values are means ± SD for 4–6 animals.
[b]Significantly different from control.
[c]Significantly different from EPA ($P < 0.05$). For other abbreviations see Table 1.

DISCUSSION

In the present experiments, the effects of hypolipidemic doses of different PUFA on the antioxidant status were investigated. Rats were fed EPA, DHA, and different EPA derivatives, that is, EPA methylated or ethylated at the 2- or 3-position, at doses known to cause hypolipidemia. Earlier our group found that high doses of EPA (1000 mg/d/kg body wt) were necessary to obtain hypolipidemic effects and elevate fatty acid oxidation in rats (7,18). The EPA derivatives used in this study have proved to reduce plasma lipids and increase the mitochondrial and peroxisomal β-oxidation in rats at low doses (Vaagenes, H., Madsen, L., Dyrøy, E., Elholm, M., Stray-Pedersen, A., Frøyland, L., Lie, Ø., and Berge, R.K., unpublished data). Low doses (250 mg/d/kg body wt) of the EPA derivatives also increased the activity of FAO. These EPA derivatives differed in their ability to increase the FAO activity, and the most potent derivatives were 2,2-dimethyl-EPA and 3-methyl-EPA (Fig. 1A). In this low-dose experiment, where rats were fed EPA derivatives, EPA or DHA, the plasma vitamin E content (Table 1) decreased only in the groups that caused the greatest induction of FAO activity, that is, 2,2-dimethyl-EPA and 3-methyl-EPA. At high doses (1500 mg/d/kg body wt) of EPA and DHA, the plasma vitamin E content decreased concomitantly with increased peroxisomal β-oxidation, measured as enzyme activity of FAO (Table 1, Fig. 1). Furthermore, plasma vitamin A was not changed by PUFA feeding in the two experiments. As both high doses of EPA and low doses of EPA derivatives are hypolipidemic (7; and Vaagenes, H., Madsen, L., Dyrøy, E., Elholm, M., Stray-Pedersen, A., Frøyland, L., Lie, Ø., and Berge, R.K., unpublished data) and the vitamin A content is unchanged, we can conclude that the plasma contents of the lipid-soluble vitamins are not a consequence of plasma lipid concentration. In the low-dose experiment, it is therefore reasonable to believe that the primary cause of decreased vitamin E content in plasma is upregulated peroxisomal β-oxidation, not polyunsaturation or reduced plasma lipids. In relation to this, it has recently been found that administration of low doses (360 mg/d/kg body wt) of EPA or DHA, which are known not to upregulate the peroxisomal fatty acid oxidation, to rats did not change the hepatic or plasma vitamin E level (19). Although the plasma vitamin E content decreased in the low-dose experiment, we observed no increase in lipid peroxidation, measured as MDA (Table 2). However, increasing the dose of PUFA administered to rats would increase the vulnerability of the cell to the hydrogen peroxide produced during elevated peroxisomal β-oxidation. Therefore, administration of DHA at high doses, which increases the peroxisomal β-oxidation, reduces plasma vitamin E content and consequently elevates lipid peroxidation. Increased vitamin E requirement with increasing polyunsaturation (5) may explain the difference between high doses of EPA and DHA on plasma lipid peroxidation. The difference in plasma MDA in rats fed equal doses of EPA and DHA could also be due to difference in the metabolism of these two n-3 fatty acids. It has recently been shown that EPA is mainly oxidized in the mitochondria, whereas DHA is most likely oxidized by the peroxisomes (20). Furthermore, DHA is a stronger peroxisome proliferator than EPA, whereas EPA causes mitochondrial proliferation. Unlike the mitochondria, the first enzyme in peroxisomal β-oxidation, FAO, produces hydrogen peroxide (1). Moreover, as EPA seems more easily oxidized than DHA (21), we expect to find a higher concentration of DHA than of EPA in plasma. Taken together, the higher amount of peroxisome-derived radicals and the higher concentration of DHA in plasma, as well as the fact that DHA contains one additional double bond, would increase the susceptibility of DHA to lipid peroxidation compared to EPA.

That the hepatic levels of vitamin E and A were unchanged in both the low- and the high-dose experiments shows that, although the plasma vitamin E level decreased, the rat was not depleted of vitamins. This observation fits with the assumption that parenchymal cells in the liver have the ability to conserve their content of vitamin E (22). Over a 3-mon period, however, administration of EPA or DHA to rats reduces the hepatic vitamin E content (6). In the low-dose experiment, there was no change in hepatic lipid peroxidation, measured as MDA. In the high-dose experiment, however, both EPA and DHA caused elevated lipid peroxidation in liver, but the hepatic content of vitamins E, A, and C were unaffected (Tables 2 and 1, respectively). Seemingly, these vitamins were not sufficient to prevent lipid peroxidation in the liver, and this might explain why EPA, with fewer double bonds than DHA, also increased hepatic lipid peroxidation. A small increase in the hepatic vitamin A content was observed in the low-dose experiment, which was not present in the high-dose experiment. The reason for this cannot be explained.

The total SOD activity tended to increase in the hepatic peroxisomal fraction of rats fed low doses of the EPA derivative

2,2-dimethyl-EPA. Significantly induced gene expression of Mn-SOD was observed (Table 3). These effects could be a kind of cellular adaptation, i.e., a secondary effect, to oxidative stress induced by 2,2-dimethyl-EPA. At high doses of EPA or DHA the activity and gene expression of SOD remained unchanged. Oleic acid, however, tended to decrease the SOD activity in the peroxisomal fraction concomitant with decreased gene expression of both Mn-SOD and CuZn-SOD. Reaven (23) has proposed that oleic acid has antioxidant properties, and these could possibly reduce the necessity of SOD activity in rats fed oleic acid, thereby downregulating this activity. Why low doses of 2,2-dimethyl-EPA, but not high doses of EPA and DHA, seemed to affect the activity of SOD remains unclear. Perhaps this effect is due to the production of hydrogen peroxide, as 2,2-dimethyl-EPA induces the FAO-activity 2.7-fold, whereas high doses of DHA or EPA only increase the FAO-activity by 1.7- and 2.0-fold, respectively. The enzyme activities of XOX and of XDH did not change in the low- or the high-dose experiments (data not shown). As the rate of conversion of XDH to XOX seemed to be unchanged compared to the control, it is likely that the amount of superoxide produced from XOX is constant. The superoxide produced from XOX would therefore not affect the SOD activity.

The enzyme activities of glutathione peroxidase, glutathione-*S*-transferase, and glutathione reductase did not change in the low- or the high-dose experiments (data not shown). The levels of glutathione and the glutathione metabolites were not affected in plasma or liver by any of the fatty acid administrations (Tables 4; additional data not shown). This indicates that the low- and high-dose experiments did not affect the glutathione metabolism to any great extent. Thus, EPA, DHA and the EPA derivatives did not seem to weaken the capacity of glutathione as an antioxidant.

The presence of homocysteine is an independent risk factor for cardiovascular diseases (24). The mechanism does not seem to involve lipid peroxidation (25,26). Our results in the low-dose experiment confirm this, since elevated plasma homocysteine in rats fed 3-methyl-EPA (Table 4) did not cause increased lipid peroxidation (Table 2).

To conclude, increased peroxisomal β-oxidation primarily seems to cause oxidative stress in rats fed PUFA. However, the amount of accumulated PUFA in the cell membranes is the critical parameter. Therefore, low doses of lipid-lowering EPA derivatives do not affect lipid peroxidation. High doses of EPA and DHA, however, overwhelm the antioxidant defense mechanisms, resulting in elevated levels of MDA in plasma and liver, even though the activity of the hydrogen peroxide-producing FAO was higher in rats fed low doses of lipid lowering EPA derivatives than high doses of EPA and DHA. The antioxidant enzymes were not affected to any great extent in these experiments.

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