Mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism

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Abstract Fish oil polyunsaturated fatty acids and fibrate hypolipidemic drugs are potent hypotriglyceridemic agents that act by increasing fatty acid catabolism and decreasing triglyceride synthesis and secretion by the liver. A major unresolved issue is whether this hypotriglyceridemic effect can occur independent of induction of peroxisomal β-oxidation, a predisposing factor for hepatocarcinogenesis. The present study was undertaken to determine which component of fish oil, eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), is responsible for its triglyceride-lowering effect. We demonstrate that EPA and not DHA is the hypotriglyceridemic component of fish oil and that mitochondria and not peroxisomes are the principal target. Results obtained by fenofibrate feeding support the hypothesis that the mitochondrion is the primary site for the hypotriglyceridemic effect. In contrast to fibrates, EPA did not affect hepatic apolipoprotein C-III gene expression. Therefore, increased mitochondrial β-oxidation with a concomitant decrease in triglyceride synthesis and secretion seems to be the primary mechanism underlying the hypotriglyceridemic effect of EPA and fibrates in rats, rabbits and possibly also in humans. In addition, these data show that lowering of plasma triglycerides can occur independently of any deleterious peroxisome proliferation.—**Frøyland, L., L. Madsen, H. Vaagenes, G. K. Totland, J. Auwerx, H. Kryvi, B. Staels, and R. K. Berge.** Mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism. *J. Lipid Res.* 1997. **38:** 1851–1858.

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High serum levels of triglyceride-rich lipoproteins, i.e., very low density lipoprotein (VLDL), and its remnants are important risk factors for coronary artery disease (1). Serum triglycerides (TG) can be lowered either by dietary treatment with fish oils (2–5) or by pharmacological treatment with drugs of the fibrate class (6–8). Fibrate action has been partly ascribed to increased fatty acid oxidation, decreased TG synthesis and secretion, and enhanced clearance of VLDL from serum due to a down-regulation of hepatic apoC-III gene expression (9,10). In addition to lowering plasma triglycerides, n–3 fatty acids (EPA,DHA) are reported to have a number of additional beneficial effects on the cardiovascular system which include antihypertensive and antithrombotic actions (11–13). EPA and DHA are the major fatty acid constituents of fish oil and it has been assumed that both EPA and DHA are responsible for its hypotriglyceridemic activity. However, growing evidence indicates that EPA and DHA may possess different hypolipidemic properties (14–16). The availability of TG is a major driving force in the secretion of VLDL by the liver (17–20). It is thus conceivable that factors influencing TG biosynthesis and/or fatty acid oxidation may ultimately influence plasma lipoprotein levels and metabolism (21). We have recently reported that EPA is the fatty acid primarily responsible for the tyriglyceride-lowering effect of fish oil (22), but the mechanism underlying this hypotriglyceridemic effect has not yet been elucidated. In the present study we used highly purified EPA and DHA preparations to investigate the effects of these fatty acids individually on plasma lipids, as well as on the proliferation of mitochondria and peroxisomes and mRNA levels and activi-

Abbreviations: apoA-I, apolipoprotein A-I; apoC-III, apolipoprotein C-III; apoE, apolipoprotein E; CPT-I, carnitine palmitoyltransferase I; CPT-II, carnitine palmitoyltransferase II; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PPAR, peroxisome proliferator-activated receptor; TG, triglyceride; VLDL, very low density lipoprotein.

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ties of their key enzymes in rat hepatocytes. Moreover, we determined whether these fatty acids modify the expression of the apoA-I, apoC-III, and apoE genes in liver. Furthermore, the rabbit animal model, in contrast to the rat model, is regarded to reflect the human situation when fed peroxisome proliferators, i.e., a modest effect on peroxisomes (23). Therefore, we compared the effects of fibrate on key enzymes in the mitochondrial and peroxisomal β-oxidation system in these two extreme models to investigate whether a common mechanism is involved in the hypotriglyceridemic effect of fish oils and fibrates.

EXPERIMENTAL PROCEDURES

n–3 Fatty acids (as ethyl esters)

Ethyl esters of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were obtained from Pronova Biocare AS (Oslo, Norway) and Norsk Hydro AS, Research Center (Porsgrunn, Norway). The purity of EPA and DHA used in the present study was 94 and 91%, respectively. EPA contained small amounts of stearidonic acid (18:4n–3) (1.1%) and docosahexaenoic acid (22:6n–3) (1.8%) while the DHA preparation contained some arachidonic acid (20:4n–6) (1.9%), eicosatetraenoic acid (20:4n–3) (1.0%), eicosapentaenoic acid (20:5n–3) (2.3%) and heneicosapentaenoic acid (21:5n–3) (4.6%). Corn oil contained linoleic acid (18:2n–6) (51.3%), oleic acid (18:1n–9) (33.6%) and palmitic acid (16:0) (10.1%). Fenofibrate was obtained from Laboratories Fournier (Daix, France).

Animals and treatments

Male Wistar rats from Möllegaard Breeding Laboratory (Ejby, Denmark) weighing about 200 g were treated (oro-gastic intubation once a day for 3 months) with the different fatty acids at a dose of 1000 mg/day per kg body weight suspended in 0.5% CM-cellulose (vehicle). Autooxidation of the stock suspensions was minimized by the addition of 0.5% (w/v) α-tocopherol and flushed with N_2. Controls received the same dose of palmitic acid. EPA and DHA were given as their ethyl esters, and this is not the form in which they would be taken during the consumption of fish oils. However, during prolonged feeding, ethyl esters of n–3 fatty acids are highly bioavailable (24,25). The levels (mol %) of EPA and DHA in the plasma were 9.8 ± 3.7 and 10.7 ± 1.9, respectively. Animals had free access to water and R34-EWOS-ALAB grower rat maintenance chow (12.5 MJ/kg) (Ewos, Sweden) which contained the following fatty acids (mol %); 16:0 (21%), 16:1n–7 (2%), 18:0 (4%), 18:1n–9 (25%), 18:2n–6 (42%), and 18:3n–3 (6%). Rats and rabbits were treated with fenofibrate (.05%, wt/wt, mixed in chow) for 5 and 10 days, respectively. The overall food consumption and weight gain were not changed in any experiment. Rats fed fenofibrate showed an increased liver weight but this was not observed in fenofibrate-treated rabbits. At the end of the experiment, the rats were fasted overnight and then were anesthetized by subcutaneous injection of Hypnorm Dormicum® (fluanisone-fentanylmidazolam 0.2 $ml \cdot g^{-1}$ body weight). Cardiac puncture was performed to obtain blood samples in EDTA-Vacutainers. The protocol was approved by the Norwegian State Board of Biological Experiments with Living Animals.

Lipid analysis

Plasma was prepared from whole blood by centrifugation at 1000 *g* for 10 min. VLDL was prepared by sequential ultracentrifugation (26), VLDL was taken as the <1.006 g/ml density fraction. Analysis of triglyceride was carried out with the Biopak triglyceride enzymatic kit (Bitrol, France).

Morphology

Parts of the ventral lobe of the liver were dissected into small pieces and fixed and prepared for transmission electron microscopy (EM) as described earlier (27). Morphometric analysis of EM micrographs (8000×) was carried out on randomly selected hepatocytes from all areas of the liver lobule using a Leitz ASM 64 K.

RNA analysis

Total cellular RNA was isolated from liver tissue by the guanidinium–thiocyanate method described by Chomczynski and Sacchi (28). Northern, dot, and slot-blot hybridizations were performed as described previously (29,30). The DNA probes were purified fragments of cloned rat genes: 2,4-dienoyl-CoA reductase: 632 bp Pst I/Eco RI insert in pGEM-4Z was kindly provided by Dr. K. Hiltunen, Umeå University, Finland; 28 S rRNA: 1.4 kb Bam HI fragment of pA. Plasmid pMJ 125, encoding partial cDNA for rat fatty acyl-CoA oxidase was kindly provided by Dr. T. Hashimoto (Shinsu University, Shinsu, Japan). DNA fragments were labeled by random priming using the oligolabeling technique of Feinberg and Vogelstein (31), resulting in specific activities ranging from 0.8 to 5×10^9 cpm/mg DNA. The rat apoA-I, apoC-III, and apoE cDNA probes are described previously (10,29). All probes were labeled by random primed labeling (Boehringer Mannheim). Filters were hybridized to 1×10^6 cpm/ml of each probe as described (29). They were washed in 300 ml of 75 mM NaCl, 7.5 mM sodium citrate, pH 7.4, and 0.1% SDS for 10 min at room temperature and twice for 30 min at

TABLE 1. Effects of palmitic acid (control), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and corn oil supplementation for 12 weeks on plasma and VLDL triglycerides and volume fractions of mitochondria and peroxisomes in rat liver hepatocytes

Group	Plasma Triglycerides		Volume Fraction		
	Total	VLDL	Mitochondria	Peroxisomes	Ratio
	mM		%		
Control	1.60 ± 0.28[a]	1.36 ± 0.17[a]	15.7 ± 1.4[a]	1.1 ± 0.1[a]	14.3[a]
EPA	1.02 ± 0.26[b]	0.95 ± 0.31[b]	21.8 ± 2.3[b]	1.2 ± 0.1[a]	18.2[b]
DHA	1.71 ± 0.27[a]	1.48 ± 0.23[a]	17.2 ± 1.6[a]	1.9 ± 0.2[b]	9.1[c]
Corn oil	1.83 ± 0.36[a]	1.68 ± 0.20[a]	11.1 ± 1.2[c]	1.1 ± 0.1[a]	10.1[c]

Plasma lipid values represent means ± SD (n = 6/group). The morphometric analysis was performed on six micrographs from each animal (n = 4/group). Values are expressed as means ± SD. Ratio denotes the ratio between mitochondrial and peroxisomal volume fractions. Means in a column with a different superscript are significantly different (ANOVA, $P < 0.05$).

65°C and subsequently exposed to X-ray film (Kodak X-OMAT-AR, Eastman Kodak Co., Rochester, NY). Autoradiograms were analyzed by quantitative scanning densiometry (Bio-Rad GS670 Densiometer) as described (29,30).

Preparation of total liver homogenates, subcellular fractions and enzyme activities

Total homogenates of the liver were prepared by homogenizing weighed portions of the individual organs in ice-cold sucrose solution containing 0.25 mol/L sucrose in 10 mM HEPES buffer and 1 mM EDTA, pH 7.4, as previously described (32). Mitochondrial 2,4-dienoyl-CoA reductase activity was measured according to Kunau and Dommes (33) with some minor modifications. Briefly, the assay medium (1.0 ml) contained 0.01% Triton X-100, 50 mM KH_2PO_4 (pH 7.4), 0.1 mM NADPH, and 100 µg protein. The reaction was started by addition of 100 µM 2,4-dienoyl-CoA substrate. The assay was run in duplicate and performed under conditions where product formation was linear with respect to time and the amount of protein. Carnitine palmitoyltransferase(CPT)-I and II were analyzed as described by Bremer (34) and fatty acyl-CoA oxidase activity was determined according to Small, Burdett, and Connock (35).

Statistical analysis

The data are presented as mean ± standard deviation (SD) from 4 or 6 animals. Treatment means were compared by one-way analysis of variance or *t*-test when appropriate.

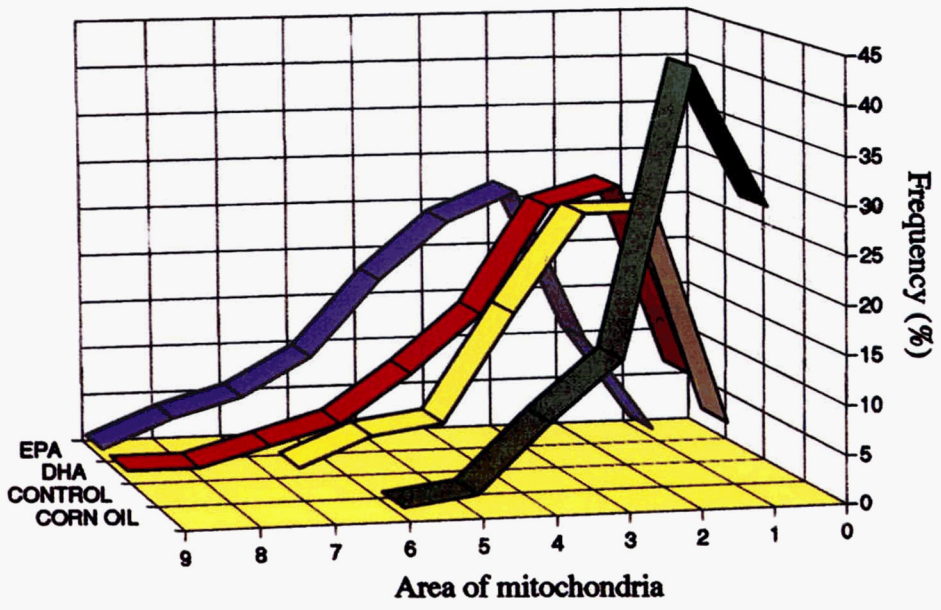

Fig. 1. Frequency distribution of mitochondrial area in hepatocytes of experimental and control rats. Numbers on the abscissa reflects a class of mitochondria with class-width 0.1 µm².

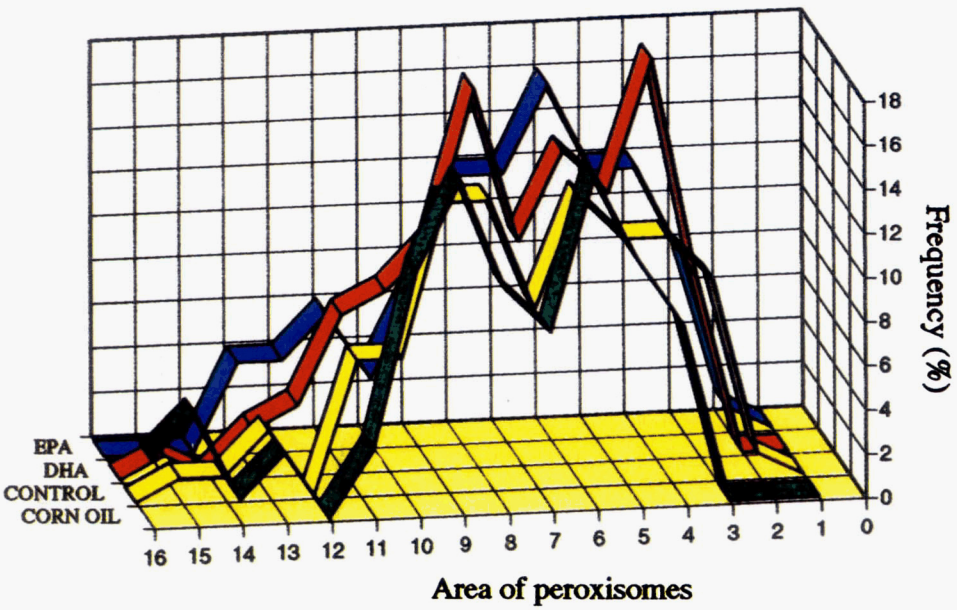

Fig. 2. Frequency distribution of perixosomal area in hepatocytes of experimental and control rats. Numbers on the abscissa reflects a class of peroxisomes with class-width 0.083 μm^2.

RESULTS

Feeding rats EPA for 12 weeks resulted in a reduction of plasma triglycerides (TG) and very low density lipoprotein(VLDL)-TG with a concomitant increased volume fraction of mitochondria (**Table 1**) and a shift towards larger mitochondria (**Fig. 1**). In contrast, DHA had no effect on plasma or VLDL-TG and did not affect the mitochondria but increased the volume fraction of peroxisomes and thus acted as an peroxisome proliferator (Table 1, **Fig. 2**). Mitochondrial β-oxidation accounts for more than 90% of total fatty acid oxidation under normal conditions and this is reflected by the high relative volume fraction of this organelle compared to peroxisomes in the liver (Table 1). Assuming that one mitochondrium and one peroxisome possess the same capacity for β-oxidation, a doubling of the mitochondrial system would theoretically require a 9-fold increase of the peroxisomal system. As indicated under Table 1, EPA feeding led to a 1.3-fold increase in the mitochondrial volume fraction compared to DHA feeding. Furthermore, the ratio between the mitochondrial and peroxisomal volume fractions is about 14.3 in controls, whereas in rats fed EPA and DHA these ratios are 18.2 and 9.1, respectively (Table 1). This indicates that EPA feeding diverts fatty acids to mitochondrial β-oxidation whereas DHA feeding affects the less efficient pathway, i.e., the peroxisomal β-oxidation system. Morphometric measurements of randomly selected hepatocytes revealed that the volume fraction occupied by fat vacuoles decreased in rats fed EPA compared to controls and corn oil-treated rats by 45% and 51%, respec-

TABLE 2. Effects of palmitic acid (control), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and corn oil supplementation for 12 weeks on hepatic fat droplets, mitochondrial 2,4-dienoyl-CoA reductase, and fatty acyl-CoA oxidase activities and mRNA levels

	Hepatic Fat Droplets	Mitochondrial 2,4-Dienoyl-CoA Reductase		Fatty Acyl-CoA Oxidase	
Group	Areal Fraction (%)	nmol/min/mg protein	mRNA levels	nmol/min/mg protein	mRNA levels
Control	0.82 ± 0.02^a	2.1 ± 0.2^a	1.0 ± 0.1^a	18.3 ± 2.5^a	1.0 ± 0.1^a
EPA	0.45 ± 0.06^b	3.1 ± 0.4^b	2.0 ± 0.3^b	29.5 ± 2.1^b	1.5 ± 0.2^b
DHA	1.15 ± 0.08^c	2.5 ± 0.3^a	1.3 ± 0.2^a	30.5 ± 4.2^b	1.6 ± 0.1^b
Corn oil	0.88 ± 0.03^a	2.2 ± 0.2^a	1.1 ± 0.1^a	20.3 ± 2.6^a	1.1 ± 0.1^a

Hepatic fat droplets, mitochondrial 2,4-dienoyl-CoA reductase, and fatty acyl-CoA oxidase activities were measured (n = 6/group). Values are expressed as means ± SD. Means in a column with a different superscript are significantly different (ANOVA, $P < 0.05$).

TABLE 3. Influence of palmitic acid (control), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and corn oil on rat liver apolipoprotein mRNA levels

Group	ApoA-I	ApoC-III	ApoE
Control	100 ± 39	100 ± 10	100 ± 10
EPA	99 ± 27	105 ± 10	97 ± 11
DHA	91 ± 12	91 ± 10	97 ± 14
Corn oil	82 ± 18	98 ± 18	95 ± 19

Animals (n = 6/group) were treated with palmitic acid (control), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and corn oil for 3 months. RNA was extracted from livers and apolipoprotein mRNA levels were measured as described under Materials and Methods. Values are expressed as mean ± SD in arbitrary units taking the mean of the control group as 100.

tively (**Table 2**). By contrast, DHA increased the volume fraction of fat droplets by 40% and 31% compared to hepatocytes from control and corn oil-fed rats, respectively (Table 2). Treatment of rats with EPA and DHA resulted in a significant increase in the fatty acyl-CoA oxidase mRNA levels and activity compared to corn oil-treated rats and controls (Table 2). In contrast to DHA feeding, treatment of rats with EPA resulted in a significant induction of rat liver gene expression and activity of mitochondrial 2,4-dienoyl-CoA reductase (Table 2), a marker enzyme involved in β-oxidation of unsaturated fatty acids. Prolonged EPA and DHA feeding did not affect hepatic apoC-III gene expression (**Table 3**).

In contrast to rats, rabbits, similar to humans, are much less responsive towards peroxisome proliferators. We therefore compared the effects of fibrates on mitochondrial and peroxisomal fatty acid oxidation between these two animal models. After 5 days treatment of rats with fenofibrate, plasma and hepatic triglyceride levels were reduced by 33% and 63%, respectively, compared to controls. Both mitochondrial and peroxisomal β-oxidation were increased by fenofibrate treatment, but the effect was more pronounced for the peroxisomes (**Table 4**). In rabbits fed fenofibrate, mitochondrial carnitine palmitoyltransferase(CPT)-I and II activities were increased by 6.5-fold and 3.8-fold, respectively, whereas the peroxisomal fatty acyl-CoA oxidase activity increased only 2.0-fold (**Table 5**). Comparing the effects of fenofibrate on the mitochondrial and peroxisomal β-oxidation capacities in rats and rabbits shows that the increase in mitochondrial β-oxidation capacity is more pronounced in the rabbit model, thereby indicating that the fatty acids were diverted towards the mitochondrial β-oxidation system (Tables 4 and 5).

DISCUSSION

The data presented in this study demonstrate that EPA, and not DHA, is the fatty acid primarily responsible for the triglyceride-lowering effects of fish oil. This is in agreement with a recent study in humans (36). Increased triglyceride (TG) clearance due to mitochondrial proliferation, with a concomitant increased mitochondrial fatty acid oxidation capacity, seems to be the mechanism underlying the hypotriglyceridemic effect of EPA. This will imply that the increased mitochondrial β-oxidation may direct free fatty acids away from TG synthesis with a concomitant decrease of TG secretion. EPA feeding led to a decrease in hepatic fat droplets whereas DHA, which seems to divert fatty acids towards the peroxisomal β-oxidation system, actually increased the volume fraction of hepatic fat droplets. The data suggest that DHA, and not EPA, is the fatty acid primarily responsible for the observed peroxisome-proliferating effect of fish oil. However, DHA, which was a peroxisome proliferator, did not affect the plasma triglyceride levels.

Mitochondria and peroxisomes are vital for sustaining life, but evolutionarily these organelles have developed distinct metabolic features. Mitochondria are more abundant than peroxisomes in most animal cells and under normal conditions oxidize more than 90%

TABLE 4. Effect of fenofibrate on plasma triglyceride levels and mitochondrial and peroxisomal β-oxidation in rats

		Mitochondrial β-Oxidation	Peroxisomal β-Oxidation	
Group	Plasma Triglycerides	Palmitoyl-L-Carnitine	Fatty Acyl-CoA Oxidase	Hepatic Triglycerides
	mM	*nmol/min/mg protein*		*μ mol/g liver*
Control	1.80 ± 0.20	1.47 ± 0.20	2.9 ± 0.1	12.97 ± 1.17
Fenofibrate	1.20 ± 0.10[a]	2.41 ± 0.19[a]	6.5 ± 0.2[a]	4.81 ± 0.28[a]

Plasma triglycerides, mitochondrial β-oxidation with palmitoyl-L-carnitine and palmitoyl-CoA as substrates, and fatty acyl-CoA oxidase activities were measured (n = 6/group). Values are expressed as means ± SD. Rats were fed fenofibrate (125 mg/day/kg body weight suspended in 0.5% CM-cellulose) for 5 days whereas controls received CM-cellulose.

[a]Significantly different from control ($P < 0.01$, paired *t*-test).

TABLE 5. Effects of fenofibrate on hepatic carnitine palmitoyltransferase (CPT) I and II and fatty acyl-CoA oxidase activities in rabbits

Group	CPT I	CPT I + 5 μM Malonyl-CoA	CPT II	Fatty Acyl-CoA Oxidase
		nmol/min/mg protein		
Control	0.78 ± 0.10	0.50 ± 0.15	3.11 ± 0.90	5.95 ± 1.11
Fenofibrate	5.08 ± 1.28[a]	3.02 ± 0.87[a]	11.71 ± 2.09[a]	12.07 ± 2.13[a]

Hepatic carnitine palmitoyltransferase I and II and fatty acyl-CoA oxidase activities were measured (n = 6/group). Values are expressed as means ± SD.

[a]Significantly different from control ($P < 0.001$, paired *t*-test).

of long-chain fatty acids (37,38). In the last two decades peroxisomes have been extensively studied with respect to hypolipidemic drugs and it is generally believed that the lipid-lowering effect is associated with peroxisome proliferation, i.e., hypolipidemic peroxisome proliferators.

Peroxisome proliferation is a pleiotropic cellular response to a range of chemical compounds (39) involving the peroxisome proliferator-activated receptor (PPAR) (40–42). This phenomenon is especially seen in rodents and has been causally linked to hepatocarcinogenesis (43,44). However, humans are less responsive and fenofibrate therapy for 2 years revealed no increase in peroxisomes (45), whereas clofibrate treatment had only a marginal effect (46). The induction of peroxisome proliferation in humans resulting from therapy with hypotriglyceridemic drugs appears doubtful therefore and it should be noted that peroxisome populations in human liver vary considerably in number and volume densities, complicating the evaluation of peroxisome numbers (39). The data presented in this paper unequivocally demonstrate that the hypotriglyceridemic action is linked to an increased mitochondrial β-oxidation, and can be dissociated from a peroxisome proliferative response.

Interestingly, fenofibrate seems to operate by the same mechanisms as EPA, i.e., by increasing the mitochondrial β-oxidation capacity. In the rat model, the mitochondrial β-oxidation increased significantly, albeit not to the same extent as the peroxisomal β-oxidation system. In the rabbit model, however, the increase in the mitochondrial β-oxidation was more evident than in the peroxisomal β-oxidation. This is an interesting finding as fibrates have a modest effect on peroxisome proliferation in humans but possess a hypotriglyceridemic effect (6–8,45). Increased lipoprotein lipase activity and down-regulation of apoC-III have been causally linked to a more efficient clearance of triglyceride-rich lipoproteins from plasma after treatment with fibrates (9,10). In light of our findings, this effect on apoC-III concomitant with an increased mitochondrial fatty acid oxidation may explain why fibrates are more potent than fish oils (EPA) in lowering serum triglycerides.

In conclusion, this study demonstrates that EPA, and not DHA, is the hypotriglyceridemic component of fish oil. EPA acts as a mitochondrial proliferator and increases the mitochondrial β-oxidation capacity. In contrast, prolonged DHA feeding increases peroxisomal β-oxidation, but has no effect on mitochondrial β-oxidation or on serum triglycerides. The hypotriglyceridemic action of nutritional (such as fish oil) and pharmacological agents (such as fibrates) can therefore be dissociated from any deleterious effects from peroxisomal proliferation.

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