Serum lipids, hepatic glycerolipid metabolism and peroxisomal fatty acid oxidation in rats fed $\omega-3$ and $\omega-6$ fatty acids

Arild C. RUSTAN,* Erling N. CHRISTIANSEN and Christian A. DREVON

Institute for Nutrition Research, University of Oslo, Oslo, Norway

Rats were fed, for 3 weeks, high-fat (20 % w/w) diets containing sunflower-seed oil, linseed oil or fish oil. Chow-fed rats were used as a low-fat reference. The high-fat diets markedly reduced non-fasting-rat serum triacylglycerol as compared with the low-fat reference, and the highest reduction (85 %) was observed with the fish-oil group, which was significantly lower than that of the other high-fat diets. The serum concentration of phospholipids was significantly reduced (30 %) only in the fish-oil-fed animals, whereas serum non-esterified fatty acids were reduced 40–50 % by both the fish-oil- and linseed-oil-fed groups. The liver content of triacylglycerol showed a 1.7-fold increase with the fish-oil diet and 2–2.5-fold with the other dietary groups when compared with rats fed a low-fat diet, whereas the hepatic content of phospholipids was unchanged. Peroxisomal fatty acid oxidation (acyl-CoA oxidase) was 2-fold increased for the rats fed fish oil; however this was not significantly higher when comparison was made with rats fed the linseed-oil diet. There was no difference in phosphatidate hydrolysis (microsomal and cytosolic fractions) among animals fed the various diets. Acyl-CoA:diacylglycerol acyltransferase activity was increased by all high-fat diets, but the fish-oil-diet-fed group showed a significantly lower enzyme activity than did rats fed the other high-fat diets. A linear correlation between acyl-CoA:diacylglycerol acyltransferase activity and liver triacylglycerol was observed, and the microsomal enzyme activity was decreased 40–50 % by incubation in the presence of eicosapentaenoyl-CoA. CoA derivatives of arachidonic, linolenic and linoleic acid had no inhibitory effect when compared with the control. These results indicate that dietary fish oil may have greater triacylglycerol-lowering effect than other polyunsaturated diets, owing to decreased triacylglycerol synthesis caused by inhibition of acyl-CoA:diacylglycerol acyltransferase. In addition, increased peroxisomal fatty acid oxidation and decreased availability of non-esterified fatty acids could also contribute by decreasing the amounts of fatty acids as substrates for triacylglycerol synthesis and secretion.

INTRODUCTION

Fish oils are enriched in long-chain polyunsaturated fatty acids of the $\omega-3$ series, especially eicosapentaenoic acid (EPA; $C_{20:5,\omega-3}$) and docosahexaenoic acid ($C_{22:6,\omega-3}$). Dietary intake of these fatty acids is effective in lowering plasma triacylglycerol [1,2]. Metabolic experiments in humans and rats have indicated that these fatty acids inhibit the production of very-low density lipoproteins (VLDL) by the liver [3]. The mechanism for this effect has been further evaluated by using cell cultures [4,5], and results indicate that decreased triacylglycerol synthesis is a major cause for the reduced secretion of VLDL triacylglycerol.

There have been attempts to identify points of regulatory potential in the esterification sequence. Glycerol-3-phosphate acyltransferase (EC 2.3.1.15), catalysing the initial esterification step, could not be identified as mediating the observed effects of fish oil [6]. Furthermore, it has been shown that feeding fish oil to rats reduces the plasma concentration of triacylglycerol and causes accumulation of triacylglycerol in the liver when compared with low-fat-diet-fed rats. The reduced plasma triacylglycerol correlated with decreased activity of phosphatidate phosphohydrolase (EC 3.1.3.4) [7].

We have previously shown that EPA decreases synthesis and secretion of triacylglycerol in cultured rat hepatocytes. This short-term effect was probably caused by inhibition of acyl-CoA:1,2-diacylglycerol acyltransferase (EC 2.3.1.20), an enzyme catalysing the last esterification step forming triacylglycerol [8]. This is the only enzyme exclusively involved in triacylglycerol synthesis and therefore has a considerable potential for specific

regulation [9,10]. The present investigation was undertaken to further explore the long-term effects of feeding a fish-oil diet on hepatic lipid metabolism, especially on synthesis of triacylglycerol and phospholipids. We have compared a diet enriched in fish oil with other high-fat diets containing $C_{18:3,\omega-3}$ or $C_{18:2,\omega-6}$ fatty acids. Chow-fed rats were used as a low-fat reference. It was particularly worthwhile to compare the effects of $C_{18:3,\omega-3}$ (linseed-oil diet) against $C_{20:5/22:6,\omega-3}$ (fish-oil diet) to evaluate the importance of fatty-acid chain length for glycerolipid metabolism and to evaluate the specific effects of feeding a fish-oil diet.

EXPERIMENTAL

Materials

The fish oil (Japanese) was a gift from DeNoFa and Lilleborg Fabriker A/S, Fredrikstad, Norway, and linseed-oil and sunflower-seed oil was from Rett-trading, Oslo, Norway. Vitamin and salt mixtures for the semisynthetic diets were from ICN Pharmaceuticals, Cleveland, OH, U.S.A. The standard chow diet (R3-Ewos-Alab) was delivered from Ewos AB, Södertälje, Sweden. [^{14}C]Oleoyl-CoA (58.7 Ci/mol) was obtained from New England Nuclear, Dreieich, Germany. Oleoyl-CoA, linolenoyl-CoA, palmitoyl-CoA, $L\alpha$ -phosphatidic acid, 1,2-dioleoylglycerol and horseradish peroxidase (type II) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Leuco-DCF (2,7-dichlorofluorescein) was from Eastman-Kodak, Rochester, NY, U.S.A. Other fine chemicals were from Sigma Chemical Co.

Abbreviations used: LF, low-fat; LO, linseed oil; SO, sunflower-seed oil; FO, fish oil; EPA, eicosapentaenoic acid; VLDL, very-low-density lipoprotein; LDCF, leuco-2',7'-dichlorofluorescein; ADGAT, acyl-CoA:1,2-diacylglycerol acyltransferase.

* Present address and address for correspondence: Department of Pharmacology, Institute of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, 0316 Oslo 3, Norway.

Animals and diets

Adult male Wistar rats weighing 150–160 g were purchased from Møllegaard Breeding Laboratory, Ejby, Denmark. The animals were fed a standard chow (R3-Ewos-Alab) for 5–9 days. After this period, the rats were separated into four dietary groups of six animals each, and the experimental diets were administered for 3 weeks. The composition of the semisynthetic diet was (% w/w): sucrose, 20; cornstarch, 33; casein (with 2% methionine), 20; cellulose, 1; vitamin mixture, 2.2; salt mixture, 4; dietary oils, 20. The low-fat chow diet consisted of (% w/w): crude protein, 22; pepsin-digestible protein, 21; carbohydrates, 52, and fat, 5. The composition of the different diets was: low-fat (LF) (R3-Ewos-Alab) containing 5% fat and the other experimental diets containing 20% dietary oils: linseed/sunflower (99:1) (LO, 62% C_{18:3,ω-3}), linseed/sunflower (21:79) (SO; 52% C_{18:2,ω-6}) and fish/sunflower (92:8) (FO, 20% C_{20:5} and C_{22:6,ω-3}). The total fatty acid composition of these diets is given in [11].

The rats were housed in grid-bottomed cages, two in each cage, and they had free access to food and water. The animals were subjected to a light period of 12 h (07:00 to 19:00 h), and the climatic conditions were: 24 °C with 60% relative humidity.

After 3 weeks of dietary modification the rats were killed between 09:00 and 11:00 h, and post-prandial blood and liver samples were taken for further analysis.

Preparation of subcellular fractions

The liver was excised for preparation of microsomes, peroxisomes and cytosol. Whole liver homogenate was used for measurement of triacylglycerol and phospholipid concentrations. A 15% liver homogenate was prepared at 4 °C in a 250 mM-sucrose solution, containing 10 mM-Hepes pH 7.4, by using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 750 g [2500 rev./min for 10 min in a Sorwall RC5C high-speed centrifuge (du Pont, Wilmington, DE, U.S.A.) using rotor SS34]. The postnuclear fraction was centrifuged at 5100 g (6500 rev./min) for 10 min to precipitate mitochondria. The supernatant was further centrifuged at 30900 g (16000 rev./min) for 10 min, and the pellet of this centrifugation is the light mitochondrial fraction enriched with peroxisomes. The supernatant from this step was further centrifuged at 63000 g (29000 rev./min) for 60 min in a Sorwall OTD 55B ultracentrifuge (rotor TFT 70.38) to obtain microsomes (pellet) and cytosol (supernatant). Light mitochondrial and microsomal fractions were resuspended in 250 mM-sucrose/10 mM-Hepes solution. Subcellular fractions were stored at –70 °C before analysis. Protein was determined by the method of Lowry *et al.* [12], with BSA as standard.

Enzyme assays

Acyl-CoA (palmitoyl-CoA) oxidase. This was assayed at 25 °C as described by Small *et al.* [13] and modified by F. Leighton (personal communication). This method is based on the determination of H₂O₂ production, which is coupled to the oxidation of LDCF (leuco-2',7'-dichlorofluorescein) in a reaction catalysed by exogenous peroxidase. The reaction mixture (1 ml) contained Tris/HCl (0.1 M, pH 8.5), horseradish peroxidase type II (EC 1.11.1.7; 0.08 mg), BSA (0.6 mg/ml), Triton X-100 (0.02%), FAD (0.015 mM), LDCF (0.05 mM) and protein (L-fraction) (10 µg). The reaction was started by addition of the substrate, palmitoyl-CoA (30 µM) and the enzymic reaction rate was determined by monitoring the oxidation of LDCF to 2',7'-dichlorofluorescein spectrophotometrically at 502 nm. Reaction rates were corrected for substrate blank.

Phosphatidate phosphohydrolase. This was measured essentially by the method of Mavis *et al.* [14]. Enzyme activity was assayed

at 37 °C in 200 µl of Tris/maleate buffer (0.05 M, pH 7) and microsomes (60 µg of protein). After 5 min preincubation the reaction was started by adding L-α-phosphatidate (1 mM), which was solubilized by sonication in 10% (v/v) ethanol before use. After 15 min incubation the reaction was terminated by adding 0.8 ml of a solution containing SDS (0.13%), ascorbic acid (1.25%), ammonium molybdate (0.32%) and H₂SO₄ (0.375 M). The phosphomolybdate colour was developed at 45 °C for 20 min, and absorbance was measured at 820 nm. The concentration of P_i was determined with a standard phosphate solution. All samples were corrected for non-enzymically produced phosphate by incubation without substrate.

Acyl-CoA:1,2-diacylglycerol acyltransferase (ADGAT). This was measured by the method of Coleman & Bell [15], slightly modified [8], using [¹⁴C]oleoyl-CoA and 1,2-dioleoylglycerol as substrates. The optimal concentrations of oleoyl-CoA and dioleoylglycerol were 30 and 125 µM respectively (saturation level). The microsomal protein concentration was 20 µg/ml (linear dependency), and the reaction was linear for at least 20 min (results not shown).

Enzyme activity was assayed at 23 °C in 500 µl of Tris buffer (175 mM, pH 7.8) containing BSA (15 µM), MgCl₂ (8 mM) and [¹⁴C]oleoyl-CoA (0.1 µCi/ml; 30 µM). After 5 min preincubation, microsomes (20 µg/ml) and 1,2-dioleoylglycerol (125 µM) dissolved in ethanol (10%, v/v, final concn.) were added and incubation continued for 10 min. The incubation was terminated by adding 20 vol. of chloroform/methanol (2:1, v/v). Serum (20 µl) was added to supply triacylglycerol as unlabelled carrier. After extraction [16], the residual lipids were redissolved in n-hexane and applied to t.l.c. plates. Radioactivity in triacylglycerol was measured by liquid scintillation. Eicosapentaenoyl-CoA was prepared as described by Kawaguchi *et al.* [17], and the concentration was assayed by using partially purified carnitine palmitoyltransferase as described elsewhere [18].

Chemical analysis

Serum triacylglycerol, phospholipid and non-esterified fatty acids were determined using enzymic, colorimetric methods obtained either from Bio Merieux (Marcy-l'Etoile, France) and from Boehringer Mannheim Biochemica (Mannheim, Germany) (non-esterified fatty acids).

Liver triacylglycerol and phospholipids were measured by g.l.c. as fatty acid methyl esters [19]. The lipid spots on t.l.c. plates were visualized by fluorescein, scraped into vials, redissolved in 1 ml benzene and methylated by overnight incubation in 2 ml of methanolic HCl (3 M) and 200 µl of 2,2-dimethoxypropane [20]. The mixtures were neutralized with 4 ml of NaHCO₃ (0.7 M) and extracted twice with 2 ml of n-hexane. After evaporation of the solvent, the lipids were redissolved in n-hexane, and an aliquot was injected into the gas-liquid chromatograph (Carlo Erba Strumentazione, Fractovap Series 2150), equipped with an apolar capillary column (CP Sil 19CB, length 50 m, diameter 0.2 mm), and using helium as carrier gas (flow rate 0.5 ml/min) [20]. The oven temperature was programmed to rise from 180 to 215 °C at 0.5 °C/min. Retention times and peak areas were automatically computed by a Hewlett–Packard 3390 A integrator. Identification of the individual methyl esters was performed by frequent comparisons with authentic standard mixtures analysed under the same conditions. Triheptadecanoylglycerol and diheptadecanoylphosphatidylcholine were used as internal standards for the quantification of triacylglycerol and phospholipids respectively.

Presentation of data and statistics

Unless otherwise stated, results are means ± S.D. of duplicate measurements in six animals of each group. Results were analysed

Table 1. Effect of diets on non-fasting serum and liver lipids

Results are means $\pm$ S.D. ($n = 5$ for serum lipids; $n = 6$ for liver lipids). Values without a common superscript are significantly different at $P < 0.05$.

Diet	Lipid				
	Serum (mM)			Liver (μ mol/g of liver)	
	Triacylglycerol	Phospholipids	Non-esterified fatty acids	Triacylglycerol	Phospholipids
LF	3.0 ± 0.5^a	2.2 ± 0.2^a	0.29 ± 0.08^a	4.2 ± 0.7^a	29.7 ± 2.3^a
LO	1.1 ± 0.4^b	1.9 ± 0.2^a	0.17 ± 0.02^b	10.1 ± 2.5^b	34.8 ± 5.6^a
SO	1.0 ± 0.3^b	2.2 ± 0.3^a	0.24 ± 0.05^a	11.0 ± 1.9^b	28.3 ± 2.2^a
FO	0.4 ± 0.1^c	1.5 ± 0.1^b	0.13 ± 0.02^b	6.3 ± 1.2^c	30.2 ± 2.8^a

Table 2. Effect of diets on fatty acid composition of liver triacylglycerol (a) and phospholipids (b)

Fatty acid composition was determined by g.l.c. as described in the Experimental section. Results are means $\pm$ S.D. ($n = 6$). nd, not detectable; tr, trace, amounts (less than 1 % of total fatty acids). Values without a common superscript are significantly different at $P < 0.05$.

(a)					
Fatty acid	Diet...	Triacylglycerol (μ mol/g of liver)			
		LF	LO	SO	FO
C _{18:2,ω-6}		0.78 ± 0.10^a	1.88 ± 0.29^b	5.28 ± 0.36^c	1.18 ± 0.17^a
C _{18:3,ω-3}		nd	3.17 ± 0.46^a	0.74 ± 0.08^b	0.16 ± 0.12^c
C _{20:3,ω-6}		tr	tr	tr	tr
C _{20:4,ω-6}		tr	tr	0.14 ± 0.04	tr
C _{20:5,ω-3}		tr	0.18 ± 0.12^a	0.10 ± 0.03^a	0.51 ± 0.15^b
C _{22:5,ω-3}		tr	0.13 ± 0.08^a	tr	0.27 ± 0.09^a
C _{22:6,ω-3}		0.14 ± 0.05^a	tr	tr	0.73 ± 0.25^b
Saturated		1.57 ± 0.14^a	1.98 ± 0.39	1.87 ± 0.30^a	1.59 ± 0.38^a
Monoenes		1.64 ± 0.09^a	2.61 ± 0.53	2.52 ± 0.30^b	1.79 ± 0.16^a
Polyenes					
ω -3		0.19 ± 0.10^a	3.55 ± 0.49^b	1.03 ± 0.12^c	1.69 ± 0.39^d
ω -6		0.80 ± 0.12^a	1.93 ± 0.32^b	5.52 ± 0.46^c	1.22 ± 0.18^a

(b)					
Fatty acid	Diet...	Phospholipids (μ mol/g of liver)			
		LF	LO	SO	FO
C _{18:2,ω-6}		4.54 ± 0.28^a	6.07 ± 0.51^b	4.38 ± 0.37^a	2.43 ± 0.34^c
C _{18:3,ω-3}		0.63 ± 0.03^a	0.75 ± 0.19^a	nd	nd
C _{20:3,ω-6}		0.37 ± 0.05^a	0.45 ± 0.09^a	0.21 ± 0.10^a	0.26 ± 0.09^a
C _{20:4,ω-6}		4.68 ± 0.51^a	4.15 ± 1.13^a	7.07 ± 0.53^b	3.25 ± 0.36^c
C _{20:5,ω-3}		0.33 ± 0.04^a	2.43 ± 0.37^b	0.51 ± 0.02^c	2.21 ± 0.21^b
C _{22:5,ω-3}		0.28 ± 0.06^a	0.62 ± 0.12^b	0.21 ± 0.03^a	0.66 ± 0.08^b
C _{22:6,ω-3}		1.26 ± 0.21^a	1.38 ± 0.43^a	1.07 ± 0.16^a	3.06 ± 0.34^b
Saturated		13.75 ± 0.51^a	16.09 ± 1.94^b	13.30 ± 0.73^a	16.02 ± 0.94^b
Monoenes		3.74 ± 0.61^a	2.42 ± 0.75^b	1.44 ± 0.20^c	2.05 ± 0.20^b
Polyenes					
ω -3		2.58 ± 0.10^a	5.38 ± 1.22^b	1.90 ± 0.26^a	6.20 ± 0.90^b
ω -6		9.22 ± 0.17^a	10.48 ± 1.64^a	11.50 ± 0.90^a	5.68 ± 0.70^b

by the Mann-Whitney non-parametric test (two-tailed) (MINITAB statistical program; Minitab Inc., State College, PA, U.S.A.).

RESULTS

All animals appeared healthy after the 3-week experimental period. There were no significant differences in food consumption between the high-fat-diet-fed groups, but the pellet-fed group

had a slightly higher consumption ($P < 0.05$) (in g/day): 23.9 ± 1.4 (LF), 19.1 ± 1.4 (LO), 18.4 ± 1.1 (SO) and 17.6 ± 2.3 (FO). No significant difference in body-weight gains was noted (in g): 310 ± 2 (LF), 311 ± 18 (LO), 296 ± 15 (SO) and 301 ± 24 (FO). Liver weights were also unaffected by the amount and composition of dietary fat (results not shown).

Serum and liver lipids

Intake of high-fat diets, with different amounts of ω -3 and

Table 3. Effect of diets on peroxisomal β -oxidation (acyl-CoA oxidase), phosphatidate-dependent hydrolysis and ADGAT activity in rat liver homogenates

Enzyme	Diet...	Activity			
		LF	LO	SO	FO
Acyl-CoA oxidase*		28 $\pm$ 4 ^a	44 $\pm$ 13 ^{a,b}	37 $\pm$ 11 ^a	59 $\pm$ 13 ^b
Phosphatidate hydrolysis†					
Microsomes		8.5 $\pm$ 1.2	8.6 $\pm$ 1.6	8.9 $\pm$ 0.9	7.5 $\pm$ 0.7
Cytosol		5.8 $\pm$ 0.4	8.0 $\pm$ 2.1	5.9 $\pm$ 0.9	6.7 $\pm$ 1.2
ADGAT‡		0.92 $\pm$ 0.07 ^a	1.45 $\pm$ 0.08 ^b	1.50 $\pm$ 0.15 ^b	1.22 $\pm$ 0.07 ^c

* Activities are expressed as nmol/min per mg of peroxisomal protein (light mitochondrial fraction). Results are means $\pm$ S.D. ($n = 6$). Values without a common letter are significantly different at $P < 0.01$.

† Activities are expressed as nmol of phosphate released/min per mg of protein. Results are means $\pm$ S.D. ($n = 6$). $P > 0.05$ among the various diets.

‡ Enzyme activities are expressed as nmol of [¹⁴C]triacylglycerol synthesized/min per mg of microsomal protein at 23 °C. Results are means $\pm$ S.D. ($n = 6$). Values without a common letter are significantly different at $P < 0.01$.

ω -6 fatty acids markedly reduced non-fasting serum concentration of triacylglycerol as compared with intake of the low-fat diet (LF) (Table 1). The highest reduction was observed for the fish-oil group, with a 85 % decrease in serum triacylglycerol. This decrease was significantly larger for rats fed fish oil (FO) compared with the other high-fat-diet-fed groups. The serum concentrations of phospholipids were unchanged, except for the FO group, which showed a 30 % reduction (Table 1). Serum concentration of non-esterified fatty acid was reduced by 40–50 % in animals fed on diets containing high amounts of ω -3 fatty acids (LO and FO) compared with the reference diet (Table 1).

The amount of hepatic triacylglycerol showed a 2–2.5-fold increase with the high-fat diets containing vegetable oils as compared with the low-fat reference, whereas it was 1.7-fold increased for the fish-oil diet (Table 1). The hepatic content of triacylglycerol was significantly lower (by 40 %) for the animals fed fish oil than those fed the other high-fat diets.

The content of total phospholipids in liver (Table 1) and microsomes, as well as microsomal triacylglycerol, was unchanged for the various dietary groups (results not shown).

Fatty acid composition of liver triacylglycerol and phospholipids

Fatty acid analysis of liver triacylglycerol and phospholipids indicated striking differences between the dietary groups (Table 2). The amount of linoleic acid ($C_{18:2,\omega-6}$) in triacylglycerol was highest in the SO group eating the largest amount of this fatty acid, whereas the level of α -linolenic acid ($C_{18:3,\omega-3}$) was highest in rats fed the diet containing linseed oil (LO). The content of very-long-chain ω -3 fatty acids in hepatic triacylglycerol in rats fed FO was 5 times higher than in rats fed LO.

The amount of $C_{18:2,\omega-6}$ and $C_{20:4,\omega-6}$ fatty acids in phospholipids was considerably lower in rats fed FO than in animals given the other diets. $C_{18:3,\omega-3}$ fatty acid was not detectable in hepatic phospholipids in the rats fed SO and FO, and even in the presence of large amounts of dietary $C_{18:3,\omega-3}$ (LO group), the concentration of $C_{18:3,\omega-3}$ in phospholipids is not significantly higher than chow-fed rats. With the LO diet rich in $C_{18:3,\omega-3}$, the $C_{20:5}$ and $C_{22:5}$ content was as high as with the FO diet, whereas the concentration of $C_{22:6,\omega-3}$ was considerably lower (50 %) and similar to LF. The total amount of polyunsaturated fatty acids was similar in all dietary groups.

The fatty acid composition of liver microsomal triacylglycerol and phospholipids was also measured, and revealed a similar pattern to that observed in total liver lipids (results not shown).

A nearly four-times-higher amount of ω -3 fatty acids in liver phospholipids than in liver triacylglycerol was observed with the fish-oil group (6.20 ± 0.90 versus $1.69 \pm 0.39 \mu\text{mol/g}$ of liver respectively) (Table 2). For the linseed-oil group, there was no

Fig. 1. Linear correlation between microsomal ADGAT and triacylglycerol content of rat liver

Results (taken from Tables 1 and 3) represent mean values $\pm$ S.D. Linear regression; $r = 0.98$; $P < 0.03$.

significant difference in the amount of ω -3 fatty acids in the two lipid classes (5.38 ± 1.22 versus $3.55 \pm 0.49 \mu\text{mol/g}$ of liver respectively; $P > 0.05$).

Hepatic enzyme activities

Peroxisomal fatty acid oxidation was determined by measuring the activity of the peroxisomal enzyme acyl-CoA oxidase. The activity was doubled in rats fed fish oil, compared with the low-fat reference (LF) and the ω -6-enriched diet (SO) (Table 3). There was no significant difference, however, in acyl-CoA oxidase activity between the two ω -3-fatty-acid dietary groups (LO and FO).

Phosphatidate hydrolysis (phosphate release) was measured in isolated microsomes and in cytosol. There was no significant difference ($P > 0.05$) between the various dietary groups in hydrolysis of phosphatidic acid (Table 3). In addition, no transfer of hydrolytic activity from cytosol to endoplasmic reticulum could be observed.

Microsomal acyl-CoA:diacylglycerol acyltransferase (ADGAT) activity showed an increase for the high-fat dietary groups when compared with the low-fat reference (Table 3). However, the activity was significantly lower for the fish-oil-treated rats as compared with those fed the LO and SO diets. A significant linear correlation between ADGAT activity and liver triacylglycerol was observed (Fig. 1). The effect of eicosapentaenoyl-CoA (EPA-CoA) on ADGAT activity for the low-fat (LF) and the ω -3 diets (LO and FO) was also studied. First,

Fig. 2. Effect of EPA-CoA on rat liver microsomal ADGAT for the low-fat and ω -3 diets

Microsomes were incubated with [14 C]oleoyl-CoA (0.1 μ Ci/ml; 30 μ M) and increasing concentrations of unlabelled EPA-CoA at 23 $^{\circ}$ C for 10 min. Data are given as nmol of [14 C]triacylglycerol synthesized/min per mg of microsomal protein and represent mean values from two separate experiments. Symbols: $\blacktriangle$, LF; $\bullet$, LO; $\blacksquare$, FO.

Fig. 3. Effect of different acyl-CoA derivatives on acyl-CoA:1,2-diacylglycerol acyltransferase for the low-fat and ω -3 diets

Microsomes were incubated with [14 C]oleoyl-CoA (0.1 μ Ci/ml; 30 μ M) and 20 μ M-unlabelled acyl-CoA at 23 $^{\circ}$ C for 10 min. Values are corrected for changes in specific activity by subtracting triacylglycerol formed in the presence of 20 μ M unlabelled oleoyl-CoA. Data are given as nmol of [14 C]triacylglycerol synthesized/min per mg of microsomal protein and represent means $\pm$ S.D. from two separate experiments. Symbols: $\square$, Control; $\blacksquare$, C_{20:5}-CoA (EPA-CoA); $\boxplus$, C_{20:4}-CoA (arachidonyl-CoA); $\boxminus$, C_{18:3}-CoA (linolenoyl-CoA); $\blacksquare$, C_{18:2}-CoA (linoleoyl-CoA).

microsomes were incubated in the presence of increasing concentrations of EPA-CoA (Fig. 2). Triacylglycerol formation was decreased by increasing concentrations of EPA-CoA for the diets examined. Second, the activity of ADGAT was also measured in

the presence of different unlabelled acyl-CoA derivatives (Fig. 3). For all dietary groups, incubation with EPA-CoA decreased enzyme activity by 40–50 %, whereas it was unchanged or slightly increased when other acyl-CoA derivatives were added to the incubation mixture (Fig. 3).

DISCUSSION

These studies show that a high intake of polyunsaturated ω -3 and ω -6 fatty acids lowers non-fasting serum triacylglycerol levels in rats compared with rats fed a low-fat high-carbohydrate diet (LF) (Table 1). The fish-oil (FO)-enriched diet gave an additional triacylglycerol-lowering effect compared with diets with polyunsaturated vegetable oils (LO and SO). The FO diet also decreased the concentration of serum phospholipids in comparison with the other dietary groups (Table 1). This hypolipidaemic effect in the non-fasting situation after fish-oil consumption is probably due to decreased formation of both chylomicrons from the intestine and VLDL from the liver [2].

All high-fat diets promoted increased liver content of triacylglycerol (Table 1). However, the liver from fish-oil-fed rats had a significantly lower amount of triacylglycerol than the other high-fat diets. Thus, even at three times higher intake of C_{18:3}, ω -3 compared with C_{20:5/22:6}, ω -3, serum as well as hepatic triacylglycerol are reduced by dietary fish oil.

From short-term experiments with cultured rat hepatocytes we know that triacylglycerol synthesis and secretion is reduced by C_{20:5} and C_{22:6}, ω -3 [5,8]. Strum-Odin *et al.* [21] also demonstrated that C_{20:5}, ω -3 might cause reduced synthesis of triacylglycerol via reduction in diacylglycerol esterification. In the present study we demonstrate that the liver content of triacylglycerol is reduced by feeding fish oil, when comparing diets containing the same amount of dietary fat (Table 1) [6,22]. We also know that there is a marked difference between long-chain ω -3 fatty acids (LO diet) and very-long-chain ω -3 fatty acids (FO diet) with respect to serum and hepatic triacylglycerol levels. An important observation in this feeding experiment is that ADGAT activity is significantly lower in fish-oil-fed animals than in animals fed vegetable oils. Furthermore, it is striking that C_{20:5}-CoA is a poor substrate for this enzyme and decreases the activity, whereas C_{18:3}-CoA, on the other hand, increases the final step of triacylglycerol synthesis (Figs. 2 and 3). It is likely that these differences between C_{20:5}-CoA and C_{18:3}-CoA are responsible for the difference in concentration of triacylglycerol in plasma and liver (Table 1; Fig. 3).

Another explanation for reduced triacylglycerol concentration in liver and serum could be that the availability of non-esterified fatty acids in the non-fasting state is decreased in animals fed fish oil. We observed a reduced serum non-esterified fatty acid concentration in rats fed linseed (LO) as well as fish oil (FO) (Table 1). It has recently been observed by Singer *et al.* [23] that fish oil most efficiently reduces serum non-esterified fatty acids in humans as compared to other polyunsaturated fatty acids. It is not clear at present why dietary fish oil decreases non-esterified-fatty-acid levels. It is possible that mobilization of fatty acids from adipose tissues is decreased or that there is a general increase in fatty acid oxidation. The last possibility is supported by the increased hepatic peroxisomal β -oxidation in the fish-oil-fed rats (Table 3) [24]. Furthermore, we have measured both mitochondrial and peroxisomal fatty acid oxidation in fish-oil-fed rats in a more recent experiment (A. C. Rustan, B. E. Hstredt & C. A. Dreon, unpublished work). We observed a 2-fold increase in peroxisomal fatty acid oxidation (H₂O₂ production), whereas no significant change in mitochondrial β -oxidation (ketone-body production) was found. It has previously been demonstrated in rats that fish-oil feeding

increases ketone production in perfused livers [6], and it has recently been observed that highly purified EPA ($C_{20:5,\omega-3}$) stimulated the activities of carnitine palmitoyltransferase, fatty acyl-CoA oxidase and peroxisomal β -oxidation [25]. Thus increased hepatic fatty acid oxidation in addition to inhibiting triacylglycerol synthesis, may also be an important factor for the decreased triacylglycerol (VLDL) secretion in the presence of very-long-chain $\omega-3$ fatty acids. In the present study, however, no significant differences between the $\omega-3$ dietary groups (LO and FO) with respect to serum non-esterified-fatty-acid concentration and hepatic peroxisomal β -oxidation were observed, although there was a tendency in favour of the FO diet providing less fatty acids for triacylglycerol synthesis (Tables 1 and 3).

We observed that phosphatidate hydrolysis (in cytosolic and microsomal fractions) was not significantly different between the high-fat-diet-fed groups, even in the presence of the fish-oil diet (Table 3). This observation is in contrast with the findings of Marsh *et al.* [7], who suggested that feeding fish-oil to rats lowered serum triacylglycerol by suppressing the activity of phosphatidate phosphohydrolase. They showed that the reduction in serum triacylglycerol correlated with the decrease in phosphatidate phosphohydrolase activity when compared with low-fat-diet-fed controls. They did not compare their results with those for other diets containing the same amount of dietary fat as we do in the present study. In addition, the content of hepatic (Table 2), as well as microsomal phospholipids was not decreased by dietary fish oil. Furthermore, a similar amount of $\omega-3$ fatty acids in liver phospholipids of animals fed linseed (LO) and fish oil (FO) was observed, despite the much higher content of $\omega-3$ fatty acids in the LO group (Tables 1 and 2). Thus our results may indicate that diacylglycerol formation is not markedly reduced by the FO diet.

Hepatic ADGAT activity was increased for all high-fat diets; however, the activity for the fish-oil-fed group was significantly lower compared with the vegetable-oil-fed groups (Table 3). This observation is in agreement with reduced hepatic triacylglycerol content for this dietary group when compared with the other high-fat-diet-fed groups (Table 1) and with the results of our short-term experiments with cultured hepatocytes [8]. Moreover, it has recently been shown that dietary fish oil decreases triacylglycerol formation relative to phospholipid in rat liver when compared with a sunflower-enriched diet [26]. Addition of EPA-CoA to microsomes when measuring ADGAT activity resulted in an inhibition of triacylglycerol formation for the low-fat diet as well as both high-fat dietary groups with $\omega-3$ fatty acids (LO and FO) (Figs 2 and 3). This observation further suggests that the activated fatty acid itself promotes reduced triacylglycerol synthesis with the FO diet, as well as reducing esterification of other acyl-CoA species. In addition, a lower amount of $\omega-3$ fatty acids was found in liver triacylglycerol with FO than in phospholipids, further suggesting that there may be a relative inhibition of hepatic triacylglycerol formation (Table 1). This enrichment of phospholipids could also be explained by increased reacylation with very-long-chain $\omega-3$ fatty acids, although increased total synthesis of phospholipids from cultured rat hepatocytes has been demonstrated after fish-oil feeding [22].

We also observed a replacement of $\omega-3$ fatty acids for $\omega-6$ fatty acids in phospholipids with the FO diet (Table 2). Moreover, in a recently published paper [11] based on this feeding experiment, reduced activities of Δ^5 -, Δ^6 - and Δ^9 -desaturases were observed with the fish-oil-diet-fed group when compared with the other high-fat-diet-fed groups. It has been observed that the lipid environment influences several membrane-bound enzymes; for instance the activity of acyl-CoA:cholesterol acyltransferase [27–29]. Despite great changes in fatty acid composition, most

studies indicate that polyunsaturated $\omega-3$ and $\omega-6$ fatty acids increase acyl-CoA:cholesterol acyltransferase activities in rat liver to the same extent, when compared with diets supplemented with saturated fat [27,30].

In conclusion, the observed triacylglycerol-lowering effects after fish-oil administration to rats, especially when compared with administration of the linseed-oil diet (LO), could be caused by decreased hepatic triacylglycerol synthesis by inhibition of diacylglycerol esterification (ADGAT activity). This may explain the additional lowering of serum triacylglycerol for the FO diet, owing to decreased triacylglycerol (VLDL) output from the liver. Furthermore, an increased incorporation of the fish-oil-specific long-chain $\omega-3$ fatty acids into liver phospholipids and an increased β -oxidation of fatty acids would also contribute to reduced triacylglycerol formation. However, the increase in β -oxidation could be secondary to a decrease in triacylglycerol synthesis, leaving more non-esterified fatty acids available for oxidation.

This work was supported from the Research Society of the Norwegian Edible Fat Producers, Family Blix Medical Fund, the Nordic Insulin Foundation, Odd Fellow Medical Research Foundation, Freia Chocolate Fabriks Medical Fund, Anders Jahres Research Foundation, Norsk Hydro A/S and Nasjonalforeningen, The Norwegian Council on Cardiovascular Disease and The Norwegian Council of Science and the Humanities. We thank Anne R. Alvestad and Joanna S. Lund for excellent technical assistance and Professor Harald Osmundsen for preparing EPA-CoA.

REFERENCES

- Sanders, T. A. B., Vickers, M. & Haines, A. P. (1981) *Clin. Sci.* **61**, 317–324
- Harris, W. S. (1989) *J. Lipid Res.* **30**, 785–807
- Nestel, P. J., Connor, W. E., Reardon, M. F., Connor, S., Wong, S. H. & Boston, R. (1984) *J. Clin. Invest.* **74**, 82–89
- Wong, S. H., Reardon, M. & Nestel, P. J. (1985) *Metab. Clin. Exp.* **34**, 900–904
- Nossen, J. Ø., Rustan, A. C., Gloppstad, S. H., Målbakken, S. & Drevon, C. A. (1986) *Biochim. Biophys. Acta* **879**, 56–65
- Wong, S. H., Nestel, P. J., Trimble, R. P., Storer, G. B., Illmann, R. J. & Topping, D. L. (1984) *Biochim. Biophys. Acta* **792**, 103–109
- Marsh, J. B., Topping, D. L. & Nestel, P. J. (1987) *Biochim. Biophys. Acta* **922**, 239–243
- Rustan, A. C., Nossen, J. Ø., Christiansen, E. N. & Drevon, C. A. (1988) *J. Lipid Res.* **29**, 1417–1426
- Haagsman, H. P., De Haas, C. G. M., Geelen, M. J. H. & Van Golde, L. M. G. (1982) *J. Biol. Chem.* **257**, 10593–10598
- Haagsman, H. P. & Van Golde, L. M. G. (1981) *Arch. Biochem. Biophys.* **208**, 395–402
- Christiansen, E. N., Lund, J. S., Rørtveit, T. & Rustan, A. C. (1991) *Biochim. Biophys. Acta* **1082**, 57–62
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Small, G. M., Burdett, K. & Connock, M. J. (1985) *Biochem. J.* **227**, 205–210
- Mavis, R. D., Bell, R. M. & Vagelos, P. R. (1972) *J. Biol. Chem.* **247**, 2835–2841
- Coleman, R. & Bell, R. M. (1976) *J. Biol. Chem.* **251**, 4537–4543
- Folch, J., Lees, M. & Sloane Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Kawaguchi, A., Yoshimura, T. & Okuda, S. (1981) *J. Biochem. (Tokyo)* **89**, 337–339
- Osmundsen, H., Neat, C. E. & Norum, K. R. (1979) *FEBS Lett.* **99**, 292–296
- Mason, M. E. & Waller, G. R. (1964) *Anal. Chem.* **36**, 583–586
- Thomassen, M. S., Christiansen, E. N. & Norum, K. R. (1982) *Biochem. J.* **206**, 195–202
- Strum-Odin, R., Adkins-Finke, B., Blake, W. E., Phinney, S. D. & Clarke, S. D. (1987) *Biochim. Biophys. Acta* **921**, 378–391
- Ribeiro, A., Mangeney, M., Cardot, P., Lorette, C., Raysigguier, Y., Chambaz, J. & Berezat, G. (1991) *Eur. J. Biochem.* **196**, 499–507
- Singer, P., Wirth, M. & Berger, I. (1990) *Atherosclerosis* **83**, 167–175

24. Yamazaki, R. K., Shen, T. & Schade, G. B. (1987) *Biochim. Biophys. Acta* **920**, 62–67
 25. Aarsland, A., Lundquist, M., Børretsen, B. & Berge, R. K. (1990) *Lipids* **25**, 546–548
 26. Yeo, Y. K. & Holub, B. J. (1990) *Lipids* **25**, 811–814
 27. Coleman, R. (1973) *Biochim. Biophys. Acta* **300**, 1–30
 28. Field, F. J., Albright, E. J. & Mathur, S. N. (1987) *J. Lipid Res.* **28**, 50–58
 29. Rustan, A. C., Nossen, J. Ø., Osmundsen, H. & Drevon, C. A. (1988) *J. Biol. Chem.* **263**, 8126–8132
 30. Spector, A. A., Kaduce, T. L. & Dane, R. W. (1980) *J. Lipid Res.* **21**, 169–179
-

Received 28 May 1991/26 September 1991; accepted 10 October 1991