Effects of dietary treatment of rats with eicosapentaenoic acid or docosahexaenoic acid on hepatic lipid metabolism

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(1) Effects of dietary treatment of male albino rats with eicosapentaenoic acid (EPA) or docosahexaenoic acid on hepatic mitochondrial lipid metabolism have been investigated. (2) Mitochondria isolated from rats given these treatments were shown to have increased ability to respire on acyl-CoA esters in the presence of malonate. This effect was expressed with most of the long-chain acyl-CoA esters used as substrates. When malonate in the incubations was replaced with malate, mitochondria from treated animals were found to exhibit diminished rates of respiration on polyunsaturated acyl-CoA esters, in particular linolenoyl-, eicosapentaenoyl- and docosahexaenoyl-CoA. This phenomenon could not be attributed to changes in activity of carnitine palmitoyltransferase I or in peroxisomal β -oxidation. (3) Uncoupled respiration on glutamate, malate or succinate was also affected by treatment with EPA. With liver mitochondria isolated from rats that had been treated with a ω -3 fatty acid in the fasted state, the respiratory rates were lower

than those observed with mitochondria isolated from control rats. Respiratory rates with mitochondria isolated from rats given the ω -3 fatty acid in the fed state was not significantly different from control rates. (4) In rats treated with EPA in the fed state, the amount of EPA incorporated into mitochondrial lipids was markedly more increased as compared to rats given ω -3 fatty acid in the fasted state. Incorporation of dietary EPA into tissue lipids was investigated, also following mildronate treatment of rats (an inhibitor of carnitine biosynthesis). (5) A hypolipidaemic effect of dietary EPA was only observed when the fatty acid was given to fed rats. Rats treated with EPA in the fasted state, in contrast, exhibited hypoglycaemia, the hypolipidaemic effects now being absent. (6) These results suggest that hypolipidaemia is most pronounced when the metabolic state favours incorporation of dietary EPA into body lipids rather than its β -oxidation, as mediated by the fed/fasted transition or by treatment with mildronate.

INTRODUCTION

Fish oils are known to have a hypolipidaemic effect both in humans and in animals [1–4]. This effect has often been associated with the presence of appreciable amounts of ω -3 fatty acids in fish oil, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Studies performed in man [5] and rats [6] have clearly shown that the hypolipidaemic effect may be attributed to these polyunsaturated fatty acids. The mechanism of the hypolipidaemic effect, however, remains obscure. EPA and DHA have been suggested to be poor substrates for the diacylglycerol acyltransferase, hence diminishing the flux of these fatty acids into triacylglycerols [7–9] and conversely favouring that into phospholipids.

High fat diets have been shown to increase rates of mitochondrial β -oxidation [10] using rat liver mitochondria or isolated rat hepatocytes, in particular with diets containing partially hydrogenated oil [11]. Dietary supplementation with EPA or DHA has, with isolated rat hepatocytes, also been demonstrated to increase cellular β -oxidation [12]. A more recent report suggests that cellular β -oxidation is stimulated in rats given EPA, while it is depressed in rats given DHA [13]. Dietary supplementation with ω -3 fatty acids has also been shown to increase hepatic peroxisomal β -oxidation [13,14], although the reported extent of stimulation varied.

In an attempt to clear up some aspects of this issue, we have undertaken a study of metabolic effects of dietary EPA and

DHA, with particular attention to hepatic mitochondrial and peroxisomal β -oxidation. We have, in parallel, investigated effects of dietary EPA on blood parameters, as well as on distribution of EPA into phospholipids in liver mitochondria and into total lipids of liver and heart. We also report results from studies designed to demonstrate whether any effects of a dietary supplement of EPA are influenced by the fatty acid being given to rats in the fed or in the fasted state, or by simultaneous treatment with mildronate (an inhibitor of biosynthesis of L-carnitine [17]).

The results suggest that dietary supplement of ω -3 fatty acids causes only a minor enhancement of hepatic peroxisomal β -oxidation, while relatively dramatic, and specific, effects on hepatic mitochondrial β -oxidation and respiration were found. The net effect appears to be a decreased flux through the citric acid cycle, while the mitochondrial ability to carry out β -oxidation is enhanced. When the level of EPA in mitochondrial phospholipids was enhanced by dietary EPA given to fed rats, or through carnitine depletion, the mitochondrial rates of respiration were found to be higher.

Our data suggest that treatment of rats with polyunsaturated fatty acids, in particular the ω -3 EPA, increases fatty acid degradation despite no marked effect on carnitine palmitoyltransferase I (CPT I; EC 2.3.1.21). Conversely, the hepatic ability to carry out lipid synthesis will be diminished, and hence will contribute to the hypolipidaemic effect of ω -3 fatty acids. Our results also suggest that treatment with ω -3 fatty acids may influence the balance between carbohydrate and lipid catabolism

Abbreviations used: CPT I, carnitine palmitoyltransferase I; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid.

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(e.g. by the fed/fasted transition). This phenomenon may be related to establishing the ω -3 fatty-acid dependence of hypolidaemia.

EXPERIMENTAL

Reagents

L-[Methyl- ^3H]carnitine was obtained from Amersham Radiochemical Centre (Amersham, Bucks., U.K.). Coenzyme A, ADP (sodium salt), NAD^+ , FAD, Hepes, dithiothreitol and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.). Palmitic, linoleic, linolenic, stearic and oleic acids were purchased from Fluka AG (Buchs, Switzerland), and were of highest purity available. EPA and DHA were from NuChek Preps (Elysian, MN, U.S.A.). L-Carnitine and mildronate (3-(2,2,2-trimethylhydrazinium)propionate) were kind gifts from Sigma-Tau (Pomezia, Italy) and Grindex (Riga, Latvia), respectively. Ethyl esters of DHA and EPA were kindly donated by Pronova A/S (Oslo, Norway). The manufacturer had assayed these preparations to be 95% pure.

Acyl-CoA esters were prepared and solutions of acyl-CoA assayed using semi-purified CPT I, as described previously [15]. All other reagents were of analytical grade or of highest purity available.

Experimental animals

Four-week-old male albino Wistar rats were purchased from Dépré (Saint-Doulchard, France). The rats were housed in stainless-steel cages and had free access to tap water and food containing 5% lipids (AO3-type pellets from U.A.R., Villemoisson/Orge, France).

Treatment of rats for studies of effects of dietary supplement of palmitate, or EPA or DHA on rates of respiration by isolated rat liver mitochondria

Rats (250 g body wt) used for studies of rates of mitochondrial respiration were given a daily dose of 1 g/kg body wt (in the fed state) of either ethyl-eicosapentaenoate or ethyl-docosahexaenoate for 10 days. Each fatty acid ester was suspended in 10% (w/w) of carboxymethylcellulose also containing 0.5% (w/v) α -tocopherol as described [13]. Control rats were given a daily dose of 1 g palmitic acid/kg body wt, also as a suspension in carboxymethylcellulose. All fatty acids were administered by gastric intubation. After 10 days of treatment, rats were anaesthetized under diethylether, and liver mitochondria isolated immediately after removal of liver. No significant differences in body weight were found between the treatment groups after 10 days of treatment.

The rats were not fasted prior to experimental use. Liver mitochondria were used immediately after isolation for measurement of respiration rates.

Treatment of rats for studies of effects of dietary supplement of EPA, given in the fed or fasted state, on lipid metabolism

In order to ensure that every rat consumed the same amount of food per day, they were allowed a daily ration of 23 g of fodder [containing 5% (w/w) of fat] throughout the experimental period. The fodder was contained in stainless-steel receptacles designed to avoid any wastage of fodder. The daily feeding-period was from 8 p.m. to 8 a.m., during which time all fodder was consumed. In addition, a first group of rats received a daily dose of 0.5 g/kg body wt of ethyl-eicosapentaenoate at 7 p.m.

(i.e. in fasted state), and a second group an identical dose at 9 p.m. (i.e. in fed state). This treatment was continued for 10 days. A third control group received a daily dose of 0.5 g of corn oil/kg body wt, which was administered at 7 p.m., also for 10 days. The ethyl-eicosapentaenoate was suspended in 10% (w/w) carboxymethylcellulose containing 0.5% (w/v) α -tocopherol, as was corn oil, and was administered by gastric intubation. Under these conditions, differences in effects of EPA should be correlated to differences in the rate of respiration between the fed and fasted states [16]. This would also be expected to influence the extent of incorporation of EPA into membrane phospholipids.

Half of the rats in each of the above three groups were given 0.3 g mildronate/kg body wt. As mildronate is known to inhibit carnitine biosynthesis [17], its administration was expected to decrease the extent of mitochondrial β -oxidation of the dietary EPA [16]. The inhibitor was mixed with fodder, and administered for 1 week prior to the start of EPA treatment, and continued until the rats were killed. Blood samples were taken from the tail vein the day before sacrifice at 6 p.m. (i.e. in fasted state), and from the dorsal aorta the following day at 8 a.m. (i.e. in fed state) under diethylether anaesthesia.

Body weights of rats receiving EPA either in fed or fasted state increased significantly as compared to rats given corn oil (by about 5 g more after the 10 day-treatment; data not shown). No significant difference was found between the two EPA-treated groups with regard to relative weights of liver or periepididymal adipose tissue (data not shown). Also the hepatic content of mitochondria did not differ between the EPA treatments (data not shown).

Measurements of mitochondrial and peroxisomal β -oxidation

Rat liver mitochondria were isolated as described previously [18]. Mitochondrial β -oxidation was measured polarographically using a Clark-type oxygen-electrode (Yellow Spring Instruments, U.S.A.). The assay contained 130 mM KCl, 25 mM Hepes, 0.1 mM EGTA, 5 mM L-carnitine, 2 mM MgCl_2 , 2 mM ADP and about 2 mg of mitochondrial protein in a final volume of 3 ml, pH 7.2. Respiration was started by addition of 30 μM acyl-CoA. Respiratory rates were measured in the presence of either 2 mM malate (to measure rates of flux through β -oxidation and the citric acid cycle) or 10 mM malonate (to measure flux through β -oxidation alone). The oxygen electrode assembly was thermostatted at 30 °C.

Parts of the mitochondrial fractions were further purified on a Percoll gradient [19]. This was carried out to diminish the presence of other organelles (peroxisomes, microsomes and lysosomes), therefore ensuring that lipids from these organelles [20] did not significantly contaminate mitochondrial lipids.

Peroxisomal β -oxidation was measured as rates of palmitoyl-CoA-dependent reduction of NAD^+ at 340 nm using rat liver homogenates which had been centrifuged at 3000 g_{av} for 1 min to remove cell debris. The assay medium contained 25 mM KP_i , 1 mM dithiothreitol, 0.5 mM NAD^+ , 0.5 mM CoA, 100 μM palmitoyl-CoA, pH 7.6.

Measurement of CPT I activity was performed as described in [21] with slight modifications [22]. Percoll-purified mitochondrial fractions (200 μg protein) were incubated in 1 ml of a mixture containing 80 mM mannitol, 75 mM KCl, 25 mM Hepes, 0.2 mM EGTA, 0.5 mM dithiothreitol, 2 mM KCN and 1% (w/v) fatty acid-free albumin, pH 7. In this assay, 40 μM palmitoyl-CoA was used alone, or in the presence of various concentrations of malonyl-CoA. The reaction was initiated by the addition of 400 nmol of L-[^3H]carnitine (1 Ci/mol). After 4 min, the reaction

was stopped by addition of 1.2 N HCl, and the acyl-[3 H]carnitine was extracted with butan-1-ol and measured in a liquid scintillation spectrometer.

Assay of lipids and proteins

Determination of triacylglycerols, phospholipids and cholesterol content in serum was performed by enzymatic methods using commercial kits (nos. 6123, 6149 and 6122, respectively, from BioMérieux (Marcy l'Etoile, France). Concentration of glucose in plasma and serum was assayed with kit no. 315-100 from Sigma Diagnostics (St. Louis, MO, U.S.A.).

Assays of proteins were carried out in the presence of SDS, using the bicinchoninic acid procedure [23].

Lipid analysis

Total lipids of tissue samples and of Percoll-purified mitochondrial fractions, to which were added 1,2,3-triheptadecanoyl-glycerol as an internal standard, were extracted by the procedure of Folch et al. [24]. All solvents contained 0.02% (w/v) of butylated hydroxytoluene as an anti-oxidant. Triacylglycerols and phospholipids were separated from total lipids on silica-gel thin layer using a mixture of hexane/diethylether/methanol/acetic acid (90:20:3:2, by vol.). Spots corresponding to the above lipid classes were scraped off the plate, and a known amount of pentadecanoic acid was added as a second internal standard.

Fatty acid analysis was determined by capillary gas liquid chromatography of methyl esters prepared using methanol-boron trifluoride. The analyses were carried out on a Becker-Packard apparatus (model 419) fitted with a 30 m $\times$ 0.3 mm i.d. glass capillary column coated with carbowax 20 M. Gas chromatography peaks were identified on the basis of retention time. Peaks were integrated and quantitated using methyl-heptadecanoate and methyl-pentadecanoate as internal standards. Integration of peaks was carried out using a Delsi model Enica 21 computing integrator (Delsi Instruments, Suresnes, France).

Statistical analysis

Results are expressed as means $\pm$ S.E.M. The significance of differences between population means was determined by using ANOVA and Fisher's test comparisons. The values in a row or column with different superscript letters are significantly different at $P < 0.05$, and values denoted by the same superscripts are not significantly different ($P > 0.05$).

RESULTS

Effects of treatment with EPA and DHA on *in vitro* mitochondrial β -oxidation

Liver mitochondria were isolated from rats treated with palmitic acid, EPA or DHA for 10 days. Using acyl-CoA esters as substrates, ADP-stimulated (state 3) rates of β -oxidation were measured polarographically with either 10 mM malonate, or with 2 mM malate, present in the incubation medium [Table 1 (A and B, respectively)]. This was carried out using the various saturated and unsaturated acyl-CoA esters indicated in the Table. Rates were measured using various concentrations of acyl-CoAs to ensure that respiratory rates were measured at a concentration giving maximal rates of β -oxidation. A concentration of 30 μ M was invariably found to be optimal.

The results presented in Table 1A show that acyl-CoA-ester-dependent respiration was enhanced following treatment with palmitate or EPA or DHA, provided malonate was included in

Table 1 Effects of dietary ω -3 fatty acids on ADP-stimulated rates of respiration obtained with rat liver mitochondria in the presence of various acyl-CoAs as substrates and with malonate or malate in the incubation medium

Tabulated values are means $\pm$ S.E.M. ($n = 6$) and are expressed as ng atom of O_2 /min per mg of mitochondrial protein. Rats were treated with EPA, DHA, palmitate or clofibrate for 10 days and compared to untreated control rats. Rates of respiration were measured using various acyl-CoA esters as substrates. Incubations were carried out in the presence of either (A) 10 mM malonate or (B) 2 mM malate. Further details are given in the Experimental section. The values within a column with the same superscript letters are not significantly different at $P < 0.05$, as determined by ANOVA and Fisher's test comparisons. All other values are significantly different. Abbreviations: 18:0, stearoyl-CoA; 18:1, oleoyl-CoA; 18:2, linoleoyl-CoA; 18:3, linolenoyl-CoA; 20:5, eicosapentaenoyl-CoA; 22:6, docosahexaenoyl-CoA.

Treatment	Rate of respiration with acyl-CoA ester (ng atom of O_2 /min per mg mitochondrial protein)					
	18:0	18:1	18:2	18:3	20:5	22:6
(A) In the presence of malonate						
Control	56 $\pm$ 8 ^a	86 $\pm$ 12 ^a	66 $\pm$ 17 ^a	57 $\pm$ 22 ^a	28 $\pm$ 6 ^a	31 $\pm$ 9 ^a
Palmitate	71 $\pm$ 12 ^a	80 $\pm$ 8 ^a	88 $\pm$ 10 ^b	84 $\pm$ 10 ^b	58 $\pm$ 14 ^b	52 $\pm$ 3 ^b
EPA	62 $\pm$ 6 ^a	91 $\pm$ 12 ^b	96 $\pm$ 5 ^c	100 $\pm$ 7 ^c	59 $\pm$ 15 ^b	56 $\pm$ 3 ^b
DHA	62 $\pm$ 18 ^a	71 $\pm$ 22 ^c	67 $\pm$ 7 ^a	79 $\pm$ 17 ^b	52 $\pm$ 19 ^b	48 $\pm$ 23 ^b
Clofibrate	62 $\pm$ 10 ^a	91 $\pm$ 18 ^b	92 $\pm$ 12 ^b	95 $\pm$ 15 ^b	62 $\pm$ 13 ^b	39 $\pm$ 11 ^{ab}
(B) In the presence of malate						
Control	148 $\pm$ 14 ^a	160 $\pm$ 20 ^a	154 $\pm$ 17 ^a	152 $\pm$ 26 ^a	114 $\pm$ 25 ^a	88 $\pm$ 17 ^a
Palmitate	105 $\pm$ 12 ^b	110 $\pm$ 13 ^b	117 $\pm$ 10 ^b	110 $\pm$ 8 ^b	98 $\pm$ 20 ^b	78 $\pm$ 12 ^b
EPA	93 $\pm$ 21 ^b	102 $\pm$ 16 ^b	93 $\pm$ 5 ^c	102 $\pm$ 11 ^b	77 $\pm$ 22 ^b	66 $\pm$ 15 ^b
DHA	90 $\pm$ 10 ^b	100 $\pm$ 12 ^b	77 $\pm$ 7 ^d	88 $\pm$ 19 ^b	72 $\pm$ 22 ^b	69 $\pm$ 16 ^b

the incubation medium. This was most marked with the polyunsaturated acyl-CoAs, but was noticeable even with stearoyl-CoA. The rates observed with mitochondria from animals treated with EPA were in general highest. Also treatment with palmitate had a similar effect, although perhaps not to the same extent as with the EPA treatment. Mitochondria from animals treated with DHA appeared to give respiratory rates somewhat lower compared to mitochondria from EPA-treated animals (Table 1A).

With malate in the medium, the situation was reversed (Table 1B), as mitochondria from rats treated with EPA and DHA gave lower rates of respiration compared to the control group. This inhibitory effect was noticed also from rats treated with palmitate, but was less marked as compared to rates obtained from animals treated with EPA or DHA. There was no detectable difference between EPA and DHA as regards suppression of malate-dependent rates of respiration (Table 1B). Also, as this effect was expressed with all fatty acyl-CoA substrates, it is clearly not exclusive to polyunsaturated substrates.

Effects of treatment with EPA given in the fasted or fed state on mitochondrial respiratory rates and on the activity of CPT I

In view of above effect of administration of ω -3 fatty acids on mitochondrial respiration, it was decided to carry out further studies of effects of administration of a ω -3 fatty acid both in the fasted state and the fed state. Practical circumstances necessitated that the study now was limited to EPA. The intention was to investigate whether the physiological state of the rat was of significance in determining effects of the dietary fatty acid. The previously described effects of dietary fatty acids on mitochondrial respiration may be mediated through changes in composition of membrane lipids. Alternatively, effects may also be mediated directly by acyl-CoA esters, or other metabolites

Table 2 Effects of dietary EPA given in the fed or fasted state on rates of FCCP-uncoupled respiration by isolated rat liver mitochondria

Tabulated values are means $\pm$ S.E.M. ($n = 5$) and are expressed as ng atom of O_2/s per mg of mitochondrial protein. Rats were given dietary EPA in the fed or fasted state, as described in the Experimental section. After 10 days of treatment, the liver mitochondria were isolated, and rates of FCCP-uncoupled respiration on glutamate/malate or on succinate were measured. Identical measurements were also carried out with mitochondria isolated from control rats (given dietary corn oil). The values within a column with the same superscript letters are not significantly different at $P < 0.05$, as determined by ANOVA and Fisher's test comparisons. All other values are significantly different. Further details are given in the Experimental section.

	Glutamate/malate	Succinate
	(ng atom of O_2/s per mg mitochondrial protein)	
Control	4.40 ± 0.07^a	3.50 ± 0.50^a
EPA given in fed state	4.85 ± 0.95^a	4.40 ± 0.40^b
EPA given in fasted state	2.97 ± 0.15^b	2.70 ± 0.07^b

Figure 1 Effects of dietary EPA given in the fed or fasted state on hepatic mitochondrial CPT I activity and its sensitivity to malonyl-CoA inhibition

Rats were given dietary EPA in the fed or fasted state, as described in the Experimental section. After 10 days of treatment, the liver mitochondria were isolated and the activity of CPT I was measured in the presence of increasing amounts of malonyl-CoA. Parallel measurements were also carried out with control rats (given dietary corn oil). Results are means ($n = 5$). Bars show S.E.M. Only the values obtained with EPA given in the fed state are significantly different from the controls at $P < 0.05$, as determined by ANOVA and Fisher's test comparisons. Further details are given in the Experimental section.

derived from the administered fatty acid. Fasting/refeeding of rats is likely to influence the balance between esterification and oxidation of a dietary fatty acid. Bearing this in mind, EPA treatment was given 2 h after commencement of feeding (fed state), or 1 h prior to commencement of feeding (fasted state).

The data shown in Table 1 were obtained with coupled mitochondria. To circumvent possible effects of treatment on ADP/ATP translocation or on phosphorylation, the subsequent studies were carried out using FCCP-stimulated respiration with citric acid-cycle substrates. This would establish whether treatment with EPA would cause effects outside β -oxidation. Also, in parallel studies, both mitochondrial β -oxidation and respiration were inhibited by cyanide, in order to study only the effects on CPT I activity.

The data presented in Table 2 show that FCCP-uncoupled respiration was depressed with mitochondria isolated from rats

Table 3 Effects of EPA given in the fed or fasted state on selected blood parameters related to lipid and carbohydrate metabolism

Tabulated values are means $\pm$ S.E.M. ($n = 5$) and are expressed as g/l of serum or plasma. Rats were given dietary EPA in the fed or fasted state. After 10 days of treatment, blood was collected at the tail vein (fasted state) and at the dorsal aorta (fed state). Serum and plasma were immediately prepared for measuring lipid parameters and glucose. All parameters were assayed in serum, excepting glucose, which also was measured in plasma. Parallel measurements were also carried out with control rats (given dietary corn oil). The values within a row with the same superscript letters (or without) are not significantly different at $P < 0.05$, as determined by ANOVA and Fisher's test comparisons. All other values are significantly different. Further details are given in the Experimental section.

	Control (g/l)	EPA given in fasted state (g/l)	EPA given in fed state (g/l)
(A) In blood of rats starved for 12 h			
Triacylglycerols	1.30 ± 0.20^a	1.24 ± 0.16^a	0.97 ± 0.08^b
Cholesterol	0.70 ± 0.06^a	0.90 ± 0.13^a	0.58 ± 0.03^b
Phospholipids	1.49 ± 0.07^a	1.36 ± 0.06^a	1.22 ± 0.03^b
Glucose in serum	0.81 ± 0.03^a	0.65 ± 0.02^b	0.81 ± 0.03^a
Glucose in plasma	0.89 ± 0.03^a	0.70 ± 0.03^b	0.87 ± 0.04^a
(B) In blood of fed rats			
Triacylglycerols	1.39 ± 0.14	1.30 ± 0.06	1.34 ± 0.06
Cholesterol	0.68 ± 0.05	0.71 ± 0.06	0.59 ± 0.04
Phospholipids	1.42 ± 0.06^a	1.34 ± 0.04^{ab}	1.26 ± 0.04^b
Glucose in serum	0.93 ± 0.01^a	0.81 ± 0.01^b	0.93 ± 0.02^a
Glucose in plasma	0.92 ± 0.02^a	0.79 ± 0.04^b	0.99 ± 0.06^a

which had been given EPA in the fasted state, as compared to rates obtained both with control mitochondria and with mitochondria isolated from rats given EPA in the fed state. This was observed using both glutamate/malate and succinate as substrates. With mitochondria from rats given EPA in the fed state, however, a higher rate of respiration was observed compared to the rates obtained with control mitochondria, although this was only significant using succinate as substrate (see Table 2).

The activity of CPT I was also affected by the physiological state at the time of treatment with EPA (Figure 1). When EPA was given in the fed state the activity of the transferase was consistently decreased by approx. 20%, whereas in rats given EPA in the fasted state the activity was not significantly different from that in control mitochondria (see rates obtained in absence of added malonyl-CoA; Figure 1). The sensitivity of CPT I to inhibition by malonyl-CoA was not altered by these treatments (Figure 1).

Effects of treatment with EPA given in fasted or fed state on blood parameters and on lipids in tissues

Analysis of blood samples taken from rats starved for 12 h showed that treatment of rats with EPA was only hypocholesterolaemic and hypotriglyceridaemic when EPA had been given in the fed state (Table 3A). When EPA was given in the fasted state, serum triacylglycerols were unchanged, while serum cholesterol increased. The amount of serum phospholipids did not vary markedly, but was somewhat decreased in the fed state (Table 3A). Relatively profound hypoglycaemia was observed when EPA had been given in the fasted state (Table 3A).

Blood samples collected after the animals had been allowed fodder (Table 3B), however, showed a mild hypoglycaemia in rats given EPA in fasted state, while those given EPA in fed state exhibited normoglycaemia. Blood triacylglycerols and cholesterol were not significantly changed compared to values found in control rats, while blood phospholipids were slightly decreased, and more so in rats given EPA in the fed state (Table 3B).

Table 4 Effects of dietary EPA given in the fed or fasted state on the amounts of EPA in total phospholipids and in triacylglycerols in liver and heart tissues

Tabulated values are means $\pm$ S.E.M. ($n = 5$) and are expressed as mg of EPA in total phospholipids or triacylglycerols per g of tissue. Rats were given dietary EPA in the fed or fasted state, as described in the Experimental section. After 10 days of treatment, total phospholipids and triacylglycerols were isolated from liver and heart tissues, and the amounts of EPA in these lipid classes were measured using CGLC. Parallel measurements were also carried out with control rats (given dietary corn oil). The values within a column with the same superscript letters are not significantly different at $P < 0.05$, as determined by ANOVA and Fisher's test comparisons. All other values are significantly different. Further details are given in the Experimental section.

	Amount of EPA (mg of EPA/g wet wt)			
	Phospholipids		Triacylglycerols	
	Liver	Heart	Liver	Heart
Control	0.10 $\pm$ 0.01 ^a	0.02 $\pm$ 0.01 ^a	0.026 $\pm$ 0.004 ^a	0.004 $\pm$ 0.001 ^a
EPA given				
In fed state	1.02 $\pm$ 0.19 ^b	0.20 $\pm$ 0.04 ^b	0.176 $\pm$ 0.044 ^b	0.032 $\pm$ 0.004 ^b
In fasted state	0.52 $\pm$ 0.12 ^c	0.12 $\pm$ 0.02 ^c	0.168 $\pm$ 0.055 ^b	0.028 $\pm$ 0.004 ^b

Table 5 Effects of treatment with mildronate together with EPA on the distribution of EPA in total phospholipids and triacylglycerols in liver and on triglyceridaemia of rats given EPA in the fed or fasted state

Tabulated values are means $\pm$ S.E.M. ($n = 4$). EPA is expressed as a molar percentage of total fatty acids of phospholipids and triacylglycerols extracted from liver tissue. Triglyceridaemia is expressed as g/l of serum. A fraction of the rats given dietary EPA in the fed or fasted state for 10 days had also received mildronate, as described in the Experimental section. Animals were fed for 12 h, then anaesthetized under diethylether. Samples of blood and liver tissue were collected immediately. The values within a column with the same superscript letters are not significantly different at $P < 0.05$, as determined by ANOVA and Fisher's test comparisons. All other values are significantly different. Further details are given in the Experimental section.

EPA given	EPA (% of total fatty acids)		Triglyceridaemia (g/l)
	phospholipids	triacylglycerols	
In fed state	4.25 $\pm$ 0.52 ^a	3.65 $\pm$ 0.40 ^a	0.97 $\pm$ 0.08 ^a
In fed state + mildronate	4.60 $\pm$ 0.23 ^a	4.72 $\pm$ 1.00 ^a	0.76 $\pm$ 0.04 ^b
In fasted state	2.60 $\pm$ 0.50 ^b	2.92 $\pm$ 0.19 ^b	1.24 $\pm$ 0.16 ^c
In fasted state + mildronate	2.75 $\pm$ 0.35 ^b	4.10 $\pm$ 0.35 ^a	1.09 $\pm$ 0.06 ^b

Table 6 Effects of dietary EPA given in the fed or fasted state on the amounts of total fatty acids and EPA extracted from rat liver mitochondria

Tabulated values are means $\pm$ S.E.M. ($n = 4$) and are expressed as μ g of total fatty acids or of EPA per mg of mitochondrial protein. Rats were given dietary EPA in the fed or fasted state, as described in the Experimental section. After 10 days of treatment, the amounts of total fatty acids and EPA were measured by CGLC. Parallel measurements were also carried out with control rats (given dietary corn oil). The values within a column with the same (or without) superscript letters are not significantly different at $P < 0.05$, as determined by ANOVA and Fisher's test comparisons. All other values are significantly different. Further details are given in the Experimental section.

	Total fatty acids (μ g fatty acid/mg protein)	EPA (μ g EPA/mg protein)
Control	167 $\pm$ 10	0.9 $\pm$ 0.1 ^a
EPA given		
In fed state	170 $\pm$ 8	7.2 $\pm$ 0.8 ^b
In fasted state	158 $\pm$ 5	4.5 $\pm$ 0.6 ^c

The results shown in Table 4 illustrate the influence of physiological state on the amount of EPA in triacylglycerols and phospholipids of total liver and heart. Treatment with EPA caused an expected increase in the amount of EPA in the lipids, this increase being more pronounced in the fed state as compared to the fasted state. This effect was most marked with respect to hepatic phospholipids, whereas the change was about the same as regards triacylglycerols. When EPA was given in the fasted state, approx. 50 and 40 % less EPA were found in phospholipids of liver and heart, respectively, as compared to the values obtained from rats given EPA in the fed state (Table 4).

Effects of treatment with mildronate on triglyceridaemia and on the distribution of EPA in lipids of liver of rats given EPA in the fed or fasted state

Results presented in Table 5 demonstrate that treatment with mildronate invariably increased the amount of EPA in hepatic phospholipids and triacylglycerols. The greater change was now invariably observed in triacylglycerols both in the fasted and fed states. Rats treated with mildronate also showed an approx. 80 % decrease in tissue carnitine content in cardiac muscle, while the decrease was approx. 50 % in liver (data not shown). This is in agreement with the well-established inhibitory effect of mildronate on carnitine biosynthesis [17]. This finding suggests that incorporation of dietary EPA into liver lipids is enhanced following a decrease in the hepatic content of L-carnitine, presumably because β -oxidative capacity is limited due to a diminished pool of L-carnitine.

In animals fasted for 12 h (as in Table 3A), treatment with mildronate under the conditions described in the Experimental section was shown to decrease triglyceridaemia by 21 and 12 % in rats given EPA in the fed or fasted states, respectively (Table 5).

Effects of treatment with EPA on distribution of EPA in mitochondrial lipids, and on respiratory rates of mitochondria isolated from liver of rats given EPA in the fed or fasted state

The data presented in Table 6 show that the total amount of fatty acids in liver mitochondrial lipids was similar in rats of the two EPA groups and in controls. Also, the amount of free fatty acids in the liver did not differ significantly between treatment groups (data not shown).

Results presented in Figure 2 demonstrate that there was a remarkable correlation between the rates of respiration measured with mitochondria from EPA-treated rats and the amount of ω -3 fatty acids in mitochondrial lipids. The results indicate that as the amount of dietary EPA found in lipids increased so did respiratory rates with succinate. The values derived from control rats (i.e. rats not given EPA) clustered in the upper left corner (Figure 2), showing that high levels of ω -3 fatty acids in mitochondrial lipids is no prerequisite for enhanced rates of respiration. When animals, however, are exposed to a relatively high dietary intake of EPA, rapid incorporation of EPA, and its elongated products, into mitochondrial lipids appears essential if respiratory rates are to be maintained at maximal levels.

Effect of treatment with various fatty acids on hepatic peroxisomal β -oxidation

Treatment with DHA brought about 20 % increase in peroxisomal β -oxidation when expressed per g liver wet wt (Table 7). Treatment with palmitic acid or EPA caused no significant changes in peroxisomal β -oxidation. Also, no significant differences were found in the activity of peroxisomal β -oxidation

Figure 2 Correlation between succinate-dependent FCCP-uncoupled respiration and total content of ω -3 fatty acids in mitochondrial lipids

Rates of respiration obtained with liver mitochondria isolated from rats treated with dietary EPA in the fed (●) or fasted state (○), as described in the Experimental section. Data obtained with control rats are also presented (□). Mean values obtained with succinate have been given in Table 2. Further experimental details are given in the Experimental section.

Table 7 Effects of treatment of rats with fatty acid with palmitic acid, EPA or DHA on activity of hepatic peroxisomal β -oxidation

Tabulated values are means $\pm$ S.E.M. ($n = 5$) and are expressed as nmol of NADH formed/min per g of liver tissue of rats treated with palmitic acid diet, EPA diet or DHA diet for 10 days. Rates of peroxisomal β -oxidation were measured as rates of palmitoyl-CoA-dependent reduction of NAD⁺ at 340 nm in liver homogenates, as described in the Experimental section. The values with the same superscript letters are not significantly different at $P < 0.05$, as determined by ANOVA and Fisher's test comparisons. All other values are significantly different. Data obtained with untreated control rats are also presented.

nmol of NADH formed/min per mg of protein				
Diet ...	Control	Palmitic acid	EPA	DHA
	0.52 $\pm$ 0.05 ^a	0.45 $\pm$ 0.04 ^a	0.55 $\pm$ 0.04 ^a	0.81 $\pm$ 0.05 ^b

in livers of rats given EPA in fasted state as compared to those treated in the fed state (data not shown).

DISCUSSION

Effect of dietary fatty acids on hepatic mitochondrial β -oxidation

Available evidence suggests that mitochondrial β -oxidation is modulated by high fat diets [10], and by hypolipidaemic compounds, e.g. clofibrate [25]. With clofibrate, both quantitative and qualitative changes were demonstrated; rates of β -oxidation were increased with most fatty acid substrates, but with the polyunsaturated ones in particular. In the present studies, polarography was used for measurements of rates of β -oxidation, rather than the spectrophotometric method [26]. Polarographic measurements allow selective measurements of β -oxidation alone (malonate in the incubation medium), as well as the combined respiration due to β -oxidation followed by the complete oxidation of the acetyl group in the citric acid cycle (malate present in the incubation medium). Our studies revealed opposite effects with regard to effects of dietary fatty acids on acyl-CoA-dependent respiration depending on conditions of incubation.

When respiration was measured as β -oxidation alone (i.e. with 10 mM malonate present in the incubations), rates of oxidation was invariably stimulated with all substrates used, although this

phenomenon was most marked with polyunsaturated substrates (Table 1A). This phenomenon is not specific for treatment with EPA or DHA, because mitochondria isolated from rats treated with palmitate exhibited a similar phenomenon (Table 1A). A similar effect has previously been observed with liver mitochondria from clofibrate-treated rats [25]. Treatment with clofibrate brought about the more substantial increase in rates of β -oxidation of polyunsaturated fatty acids, as compared to monounsaturated or saturated fatty acids. In the experiments presented here, β -oxidation increased by 50–100%, depending on substrates (linolenoyl-, eicosapentaenoyl- or docosahexaenoyl-CoA gave the larger increases). This was not caused by increases in specific activity of CPT I, as this activity was not markedly altered by treatment with EPA (Figure 1). The enhanced β -oxidation is unlikely to be due to increased peroxisomal β -oxidation; a marginal increase in peroxisomal β -oxidation was observed only with rats treated with DHA (Table 7).

The inhibitory effects of a dietary supplement of fatty acids on malate-dependent respiration of acyl-CoAs were rather surprising. The results presented in Table 1B show this effect to be expressed with all substrates. These data also indicate that the effect is more marked with the acyl-CoAs giving higher rates of respiration (e.g. stearoyl-, oleoyl-, linoleoyl- and linolenoyl-CoAs). With these substrates, the degree of inhibition was about 50%. With EPA-CoA and DHA-CoA approx. 30 and 20% inhibition was observed, respectively (Table 1B). It is also apparent from the data presented in Table 1B that the extent of inhibition is less marked with mitochondria isolated from rats given palmitate, as opposed to mitochondria from rats given DHA or EPA. This effect, however, is only marked with polyunsaturated substrates.

These findings clearly suggest that the flux through the citric acid cycle is diminished following a dietary fatty-acid supplement to fasted rats. This occurs in a situation where β -oxidation is particularly enhanced, and is therefore likely to mimic the ketogenic state of the liver [16]. As shown in Table 1A, this effect was also induced by any one of the ω -3 fatty acids supplied to the animals, although DHA was clearly less efficient than both EPA and palmitate in this respect, except when EPA and DHA were substrates for β -oxidation.

The phenomenon found with FCCP-stimulated respiration on either malate/glutamate or succinate (see Table 2) cannot be directly correlated to the above observations, the major reason for this being the use of uncoupling conditions (as well as different substrates). However, the diminished rates of FCCP-uncoupled respiration found with liver mitochondria from rats treated with EPA in the fasted state, as compared to animals given EPA in the fed state (see Table 2), serve to substantiate the importance of the physiological state of the animal in determining the effect of treatment with EPA. As uncoupled mitochondria exhibit maximal rates of electron transport, treatment-dependent differences in respiratory rates may now be due to effects on enzymes which are not integral to the respiratory chain, e.g. most enzymes of the citric acid cycle. Hence the lower rates of respiration with glutamate/malate, or with succinate, observed with mitochondria from rats given EPA in the fasted state are likely to be due to effects on these enzymes. Direct alteration of properties of the respiratory chain cannot, however, be excluded.

Nevertheless, findings presented in Table 2 demonstrate that dietary treatment with a ω -3 fatty acid brings about permanent changes in mitochondrial respiratory properties, and that the nature of this change is dependent on the physiological state of the animal. The mechanism by which these changes are brought about is not clear, and will be a subject of future studies.

Stimulation of hepatic mitochondrial β -oxidation has been found in rats fed on partially hydrogenated marine oil [10] and in rats fed on oil rich in α -linolenic acid [27]. In isolated rat hepatocytes, citric acid cycle activity was inhibited by increased concentrations of palmitate [28], while β -oxidation itself was increased. This latter phenomenon may involve stimulation of gluconeogenesis [29].

EPA in tissue lipids and mitochondrial β -oxidation

The amount of EPA incorporated into tissue lipids was invariably less in the fasted state compared to the fed state (see Tables 4 and 5). In the liver, the amount of EPA found in phospholipids was lower when EPA was given in the fasted state than in the fed state, as compared to heart muscle (Table 4). This difference between fed and fasted states was much less marked with respect to triacylglycerols, and no major difference was apparent between the tissues in this respect (Table 4). On simultaneous treatment with mildronate, the amount of EPA in phospholipids and in triacylglycerols increased in both the fed and fasted states (Table 5). Now, however, in both states the increase in EPA content was most marked in triacylglycerols. This increase is presumably a consequence of diminished capacity for mitochondrial β -oxidation due to lower levels of L-carnitine in the tissue [17]. Under these conditions, it would appear that triacylglycerols provide a primary buffer for excess EPA and, perhaps, for excess fatty acids in general. The finding of major decrease in EPA in hepatic phospholipids in fasted rats (Table 5) suggests that, in the fasted/fed metabolic transition, phospholipids constitute a significant fatty acid buffer, at least in the short term. It is also striking that the more EPA was recovered in tissue lipids, the higher was the hypotriglyceridaemic effect, i.e. comparing effect of EPA given in the fed state vs. in the fasted state.

Similarly, treatment with mildronate in addition to EPA further stimulated incorporation of EPA into lipids in both fed and fasted state (Table 5), and brought about an additional decrease in blood triacylglycerols (Table 5).

Is there a relationship between amounts of EPA incorporated into mitochondrial lipids and rates of mitochondrial respiration?

The total amount of mitochondrial fatty acids per mg of mitochondrial protein did not change markedly, depending on the fasted/fed transition (see Table 6). Treatment with EPA led to about 10-fold increase in the amount of EPA in these lipids. In the fasted state this amounted to only about 60 % of that found in the fed state (Table 6).

The apparent correlation between rates of mitochondrial respiration and amount of ω -3 fatty acids in mitochondrial lipids is somewhat striking (Figure 2). Also, in control animals no such correlation is apparent (Figure 2), hence the conclusion that a dietary supplement of EPA given in the fasted state caused an altered mitochondrial metabolic state. In the fasted state, more EPA can be envisaged as being channeled in the direction of oxidative metabolism, and less into storage/structural lipids. Therefore in the fasted state, metabolites from EPA would somehow cause diminished rates of mitochondrial respiration with citric acid-cycle substrates (Table 2 and Figure 2).

The cause of the apparent inhibitory effects on the citric acid-cycle activity is not clear, but it has been suggested that long-chain fatty acids may impede flux through the citric acid cycle [30,31]. This phenomenon has also been reported with heart mitochondria [32], especially with respect to C22:1 fatty acids [33,34].

Dietary EPA, fatty acid and carbohydrate catabolism

In our experiments, there is a striking difference regarding effects of dietary EPA on blood glucose and lipids depending on the physiological state of the rat. It is only when EPA had been given in the fed state that hypolipidaemia is marked (see Table 3). Even when blood samples were taken from non-fasting rats, some hypolipidaemia is apparent. As regards blood glucose, hypoglycaemia is apparent in rats given EPA in fasted state, and this is even apparent in blood samples taken from non-fasting rats, in accordance with earlier findings [35]. EPA-dependent hypoglycaemia suggests that carbohydrate catabolism is accelerated when EPA was given to fasted animals. The hypolipidaemia (and absence of hypoglycaemia) found in blood from animals treated with EPA in the fed state, may result from diminished synthesis and export of very-low-density lipoprotein from the liver. In line with this argument the hepatic level of triacylglycerol was found not to be increased in these rats (data not shown), and this may therefore reflect increased hepatic β -oxidation (Table 1). Diminished lipolysis may also occur, as dietary EPA can inhibit lipolysis in mice [36].

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