ELSEVIER

Biochimica et Biophysica Acta 1369 (1998) 193–203

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

On the effect of 2-deuterium- and 2-methyl-eicosapentaenoic acid derivatives on triglycerides, peroxisomal β-oxidation and platelet aggregation in rats

Nina Willumsen [a], Hege Vaagenes [a], Holm Holmsen [b], Rolf K. Berge [a,*]

[a] *Department of Clinical Biology, Division of Biochemistry, Haukeland Hospital, University of Bergen, N-5021 Bergen, Norway*
[b] *Department of Biochemistry and Molecular Biology, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway*

Received 13 August 1997; accepted 1 September 1997

Abstract

A series of 2-substituted eicosapentaenoic acid (EPA) derivatives (as ethyl esters) have been synthesized and evaluated as hypolipidemic and antithrombotic agents in feeding experiments in rats. Repeated administration of purified 2-methyl-eicosapentaenoic acid and its deuterium analogues (all as ethyl esters) to rats resulted in a decrease in plasma triglycerides and high density lipoprotein cholesterol. The 2-methyl-EPA analogues were, apparently, four times more potent than EPA in inducing the triglyceride lowering effect. The 2-deuterium-2-methyl-EPA decreased plasma cholesterol level to ~40%. A moderate enlargement of the liver was observed in 2-methyl-EPA treated rats. This was accompanied with an acute reduction in the liver content of triglycerides and a stimulation of peroxisomal β-oxidation and fatty acyl-CoA oxidase activity. The results suggest that the triglyceride-lowering effect of 2-methyl-EPA may be due to a reduced supply of fatty acids for hepatic triglyceride biosynthesis because of increased fatty acid oxidation.

Platelet aggregation with ADP and A23187 was performed ex vivo in platelet-rich plasma, after administration of different doses of the EPA-derivatives for five days. EPA and 2,2-dideuterium EPA had no effect on ADP-induced aggregation, while 2-deuterium-, 2-methyl- and 2-deuterium-2-methyl EPA produced a biphasic effect, i.e. potentiation and inhibition at low (250 mg/day kg body weight) and higher doses (600–1300 mg/day kg body weight), respectively. A23187-induced platelet aggregation was affected in a similar way by feeding the 2-substituted EPA derivatives, except that 2-deuterium-2-methyl EPA had no effect relative to EPA itself and that the inhibition was far greater than that for ADP-induced aggregation (~100% inhibition with 600 mg 2-methyl-EPA/day kg body weight). The ranking order of the EPA-derivatives to affect platelet aggregation and to cause hypolipidemia was different, suggesting different mechanisms. Our observations suggest that the effects of the EPA derivatives on platelet aggregation could be related to the degree of bulkiness around C_2 and that an asymmetric substitution at C_2 caused inhibition of platelet aggregation while a symmetric

Abbreviations: A23187, divalent cationophore; ADP, adenosine diphosphate; CMC, carboxymethyl cellulose; CoA, coenzyme A; D, deuterium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GC, gas chromatograph; HDL, high density lipoprotein; MS, mass spectroscopy; NMR, nuclear magnetic resonance; O.D., optical density; PC, phosphatidylcholine; PI, phosphatidylinositol; PLC, phospholipase C; PPARα, peroxisome proliferator-activated receptor α; PRP, platelet rich plasma; VLDL, very low density lipoprotein

* Corresponding author. Fax: +47 55 973115.

PII S0005-2736(97)00211-3

substitution did not. It is suggested that the bulky, asymmetric derivatives inhibit platelet aggregation by altering platelet membrane phospholipid packing. © 1998 Elsevier Science B.V.

Keywords: Platelet aggregation; Fatty acid oxidation; Eicosapentaenoic acid; Lipid metabolism; Triglyceride

1. Introduction

Polyunsaturated fatty acids of fish origin, e.g. eicosapenta--enoic acid (EPA, 20 : 5 *n*-3) and docosahexaenoic acid (DHA 22 : 6 *n*-3), are currently being extensively investigated in laboratory and clinical science. Included among a wide spectrum of biological effects, these two fatty acids possess lipid lowering and antithrombotic properties, and several studies suggest an inverse relationship between the consumption of marine foods, rich in *n*-3 fatty acids, and the incidence of cardiovascular disease [1–4]. Fish oil affects nearly every major pathway of hepatic fatty acid metabolism. The hypotriglyceridemic effect of dietary fish oil is attributed to increased fatty acid oxidation (mitochondrial and/or peroxisomal) [5–9], inhibited lipogenesis [5,10,11] and accordingly, to low formation and secretion of triglycerides and VLDL in the liver [12–14]. Recently, we have observed that highly purified EPA stimulates mitochondrial and peroxisomal β-oxidation and carnitine palmitoyltransferase activity followed by diminished lipogenesis [8]. It is noteworthy that relatively high doses of highly purified EPA (1500 mg/day kg body weight) are necessary for the reduction of plasma triglycerides in rats. Also, in clinical studies, fairly large amounts of *n*-3 fatty acids during an extended period of time seem necessary to achieve significant results [9,15–17]. Our work with saturated 3-thia fatty acids, lipid-lowering agents with both hypotriglyceridemic and hypocholesterolemic properties, have resulted in the same triglyceride lowering effect at a dose of 150 mg/day kg body weight [18]. The hypolipidemic effect of 3-thia fatty acids is probably exerted by blocking of mitochondrial β-oxidation [19].

Long-chain polyunsaturated fatty acids are relatively poorly oxidized by the mitochondrial β-oxidation system, and recently it has been shown that introduction of a methyl group in the 2-position of fatty acids is sufficient to prevent its β-oxidation in mitochondria [20]. The results from experiments with 3-thia fatty acids initiated the search for simpler derivatives of EPA that may result in hypolipidemia. We have therefore synthesized 2-methyl-derivatives of EPA with both hydrogen and deuterium (Fig. 1) in the 2-position, which might reveal a desireable lipid-lowering effect with minimal side effects.

The present data support the concept that the potency of long-chain fatty acids, saturated as polyunsaturated, as hypotriglyceridemic compounds depend on their accessibility for β-oxidation. Furthermore, we also report results from studies on aggregation of platelets from rats treated with these hypotriglyceridemic agents. Numerous studies have shown a dampening of platelet function, following the ingestion of fish and fish oils [21,22]. In the present study, platelet aggregation was used to evaluate the potency of the selected compounds as antiaggregating agents.

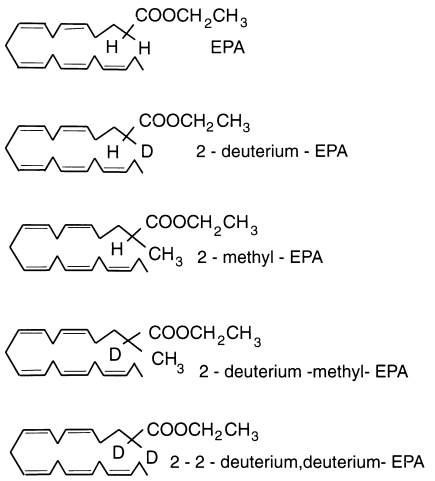

Fig. 1. Structural formula and names of synthesized fatty acid analogs of eicosapentaenoic acid ethyl esters.

2. Materials and methods

2.1. Chemicals

Palmitoyl-CoA was purchased from Sigma Chemicals, whereas palmitic acid was purchased from Aldrich-Chemie (Steinheim, Germany). Eicosapentaenoic acid ethyl ester (95% pure) was prepared at Norsk Hydro AS, Research Centre, Porsgrunn, Norway. All other chemicals were obtained from common commercial sources and were of reagent grade.

2.2. Synthesis of 2-methyl-eicosapentaenoic acid derivatives from eicosapentaenoic acid ethyl ester

A solution of BuLi in hexane (33.0 ml, 1.6 M, 0.0495 mol) was added with a syringe to diisopropyl amine (5.0 g, 0.0495 mol) in dried THF (40 ml) at −35°C while stirring. This solution was further cooled down to −70°C, and EPA ethyl ester (14.8f, 0.045 mol, 95% pure by GC) was added dropwise at this temperature for 1.5 h. The solution was kept at −70°C for a further 20 min before CH_3I (10 ml) was added in one portion. The reaction mixture was held at this temperature for 15 min and then slowly heated to room temperature.

The reaction mixture was poured into a mixture of water (100 ml) and heptane (100 ml). The organic phase was washed with water (2×75 ml), $NaHCO_3$ (75 ml), again with water (75 ml) and dried over Na_2SO_4 for 24 h. 2-Methyl-EPA ethyl ester (14.7 g, 95%) was collected after filtration and evaporated. The purity was 90% by GC. This product was further purified to 95% (GC) by prep-LC and evaporated. The purity was 90% by GC. This product was further purified to 95% (GC) by prep-LC.

MS m/z (rel. intensity): 344(3), 315(3), 290(2), 275(7), 201(20), 175(17), 119(46), 91(78), 79(100).

^{1}H NMR(300MHz) ($CDCl^3$): 0.8 (t,3H), 1.1(d,3H), 1.2 (t,3H), 1.4(m,1H), 1.55(s,1H), 1.75(m,1H), 2.05(m,6H), 2.8(m,6H), 4.05(q, 2H), 5.2(m,10H).

2-Deuterium-eicosapentaenoic acid ethyl ester was prepared in the same manner as 2-methyl-eicosapentaenoic acid ethyl ester except heavy water (10 ml D_2O) was added instead of CH_3I.

2,2-Dideuterium-eicosapentaenoic acid ethyl ester was prepared from 2-deuterium-eicosapentaenoic acid ethyl ester that was quenched once more with 10 ml D_2O.

2-Deuterium-2-methyl-eicosapentaenoic acid ethyl ester was prepared from 2-methyl-eicosapentaenoic acid which was quenched with 10 ml D_2O.

2.3. Animals and treatment

Male Wistar rats from Møllegaard Breading Laboratory, Ejby, Denmark, weight 150–180 g, were housed individually in metal wire cages in a room maintained at 12 h light–dark cycles and at a constant temperature of 20 ± 3°C. The animals were acclimatized for at least one week under these conditions, before the start of the experiment.

The EPA derivatives and EPA (all as ethyl esters) were suspended in 0.5% carboxymethyl cellulose (CMC) with 0.5% tocopherol and administered by gastric intubation at doses of 250, 600 and 1300 mg/day kg body weight in a volume of 0.7–1.0 ml once a day for five days. The control animal groups received only CMC and 0.5% tocopherol. All animals had free access to water and food. The food composition was as earlier described [23], containing 55% carbohydrate (w/w), 25% protein and 2.1% fat (w/w) and all necessary minerals and vitamins.

The body weights of the rats were measured daily. At the end of the experiments, the rats were fasted overnight and weighed. Under light halothane anaesthesia, cardiac puncture was performed to obtain blood samples (in EDTA). The livers were removed and immediately chilled on ice and weighed. Plasma was prepared from the blood samples by centrifugation at $1000 \times g$ for 10 min.

2.4. Preparation of subcellular fractions

Livers from individual rats were homogenized in an ice-cold sucrose medium (0.25 M sucrose in 10 mM HEPES buffer, pH 7.4, and 1 mM EDTA). The resulting nuclear + postnuclear fraction was taken as the total homogenate, and a light mitochondrial enriched fraction was isolated as previously described [24]. All procedures were performed at 0–4°C. The different fractions were stored at −80°, until analyzed.

2.5. Analytical methods

Protein was assayed by Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). Lipid analyses were car-

ried out by the Monotest cholesterol enzymatic kit (Boehringer Mannheim, Germany), the Monotest phospholipid enzymatic kit (Boehringer, Mannheim, Germany) and the Biopak Triglyceride enzymatic kit (Biotrol, Paris, France).

Enzymatic activities of fatty acyl-CoA oxidase [25] and peroxisomal β-oxidation (palmitoyl-CoA dependent dehydrogenase) [26] were determined as described earlier.

2.6. Platelet aggregation studies

Blood was collected by cardiac puncture into 1/10 volume of 0.22 M Na_3-citrate and centrifuged at $G_{max} = 180 \times g$ at room temperature for 15 min. The platelet-rich plasma so obtained was used for determination of platelet aggregation with A23187 (2.5 and 5 μM) and ADP (1 and 4 μM) in a Payton Dual Channel Aggregometer (200 μl samples) at room temperature and a stirring speed of 900 rpm.

2.7. Presentation of results

Variation in response from animal to animal was estimated separately for selected enzymes and lipids in the group of control and experimental animals. Data are presented as mean ± SD. Six animals in each experimental group and six controls were used. Data were statistically analyzed by Student's *t*-test.

3. Results

3.1. Body and liver weight

The rats fed normolipidemic diet supplemented with EPA, 2-methyl-EPA and their deuterium analogues gained weight per day at the same rate as controls. Food consumption was similar in each experimental group, irrespective of the dietary regime, indicating that the appetite was not affected and that the *n*-3 fatty acid analogues were well tolerated. All animals treated with fatty acids at various doses appeared healthy, had normal body weight and normal hepatic concentration of protein (data not shown) and looked and behaved normal. No hepatomegaly resulted after feeding EPA, 2-deuterium-EPA and 2,2-dideuterium-EPA even at a dose of 1300 mg/day kg body weight, whereas a moderate enlargement of the liver was observed in 2-methyl EPA treated rats (Table 1).

Table 1
Dose-dependent changes of liver weight and liver lipids in rats treated with EPA and structural analogs of 2-methyl-EPA (all as ethyl esters) for 5 days [a]

Parameters	Compound	Dose (mg/day kg body weight)			
		0	250	600	1300
Relative liver weight (g/body weight × 100%)	EPA	3.46 ± 0.04	3.54 ± 0.04	3.51 ± 0.17	3.74 ± 0.13
	2-D-EPA	3.12 ± 0.03	3.24 ± 0.12	3.14 ± 0.08	3.05 ± 0.06
	2,2-diD-EPA	3.08 ± 0.04		2.97 ± 0.02	
	2-methyl-EPA	3.29 ± 0.13	3.44 ± 0.14	3.60 ± 0.16	3.79 ± 0.08 [b]
	2-D-2-methyl-EPA	3.23 ± 0.16	3.26 ± 0.13	3.42 ± 0.31	3.60 ± 0.18
Hepatic triglycerides (μmol/g liver)	EPA	5.45 ± 1.01	5.10 ± 1.18	4.71 ± 0.89	4.80 ± 0.74
	2-D-EPA	4.37 ± 1.08	5.48 ± 0.48	6.59 ± 2.81	6.31 ± 0.84
	2,2-diD-EPA	4.51 ± 0.04			4.52 ± 0.10
	2-methyl-EPA	6.31 ± 0.75	4.58 ± 0.92	4.18 ± 0.28 [b]	4.95 ± 0.14 [b]
	2-D-2-methyl-EPA	5.83 ± 0.33	3.77 ± 0.62 [b]	4.15 ± 0.26 [b]	4.84 ± 0.35 [b]
Hepatic cholesterol (μmol/g liver)	EPA	11.05 ± 0.59	12.40 ± 1.26	12.17 ± 0.21	12.42 ± 0.41
	2-D-EPA	12.22 ± 0.58	13.12 ± 0.29	13.50 ± 0.29	13.13 ± 0.09
	2,2-diD-EPA	10.52 ± 0.26			10.90 ± 0.21
	2-methyl-EPA	12.99 ± 0.54	12.93 + 0.72	13.53 ± 0.06	13.25 ± 0.25
	2-D-2-methyl-EPA	12.56 ± 0.43	12.62 ± 0.38	12.50 ± 0.60	12.15 ± 0.84

[a] Values are expressed as means ± SD for six animals in each experimantal group.
[b] $P < 0.05$ as compared to controls.

Table 2
Dose-dependent changes of serum lipids in rats treated with EPA and structural analogues of methyl-EPA (all as ethyl esters) for 5 days [a]

Parameters	Compound	Dose (mg/day kg body weight)			
		0	250	600	1300
Serum Triglycerides (μmol/l)	EPA	0.99 ± 0.18	1.04 ± 0.28	0.86 ± 0.13	0.69 ± 0.10 [b]
	2-D-EPA	1.05 ± 0.06	1.27 ± 0.25	0.82 ± 0.17	0.95 ± 0.16
	2,2-diD-EPA	0.56 ± 0.12			0.35 ± 0.06 [b]
	2-methyl-EPA	1.08 ± 0.13	0.99 ± 0.11	0.52 ± 0.03 [b]	0.70 ± 0.08 [b]
	2-D-2-methyl-EPA	0.93 ± 0.06	0.70 ± 0.08 [b]	0.64 ± 0.20 [b]	0.58 ± 0.14 [b]
Serum Cholesterol (μmol/l)	EPA	1.88 ± 0.06	1.74 ± 0.27	1.51 ± 0.35	1.69 ± 0.15
	2-D-EPA	1.71 ± 0.18	1.52 ± 0.20	1.48 ± 0.16	1.59 ± 0.24
	2,2-diD-EPA	1.91 ± 0.19			1.64 ± 0.11
	2-methyl-EPA	1.62 ± 0.02	1.50 ± 0.14	1.41 ± 0.31	1.49 ± 0.29
	2-D-2-methyl-EPA	1.92 ± 0.08	1.77 ± 0.23	1.49 ± 0.26	1.13 ± 0.04 [b]
Serum HDL-cholesterol (μmol/l)	EPA	1.33 ± 0.02	1.36 ± 0.18	1.10 ± 0.22	1.02 ± 0.12 [b]
	2-D-EPA	1.17 ± 0.09	1.07 ± 0.08	0.98 ± 0.13	1.13 ± 0.19
	2,2-diD-EPA	1.47 ± 0.01			1.16 ± 0.05 [b]
	2-methyl-EPA	1.35 ± 0.02	1.19 ± 0.13	1.22 ± 0.22	1.08 ± 0.20 [b]
	2-D-2-methyl-EPA	1.59 ± 0.18	1.41 ± 0.21	1.29 ± 0.06	0.92 ± 0.06 [b]

[a] Values are presented as means ± SD from 6 animals.
[b] $P < 0.05$ compared to controls.

3.2. Serum and hepatic lipids

In keeping with previous observations [8,9], serum triglycerides and serum HDL-cholesterol were significantly lowered after administration of EPA at a dose of 1300 mg/day kg body weight (Table 2). Repeated administration of this *n*-3-fatty acid only marginally affected the serum cholesterol (Table 2), hepatic triglycerides and hepatic cholesterol (Table 1). It has recently been observed, however, that hypocholesterolemic effect is observed in EPA-treated rats for a longer feeding time [9]. When 2-methyl-EPA was

Table 3
Dose-dependent changes of peroxisomal β-oxidation and fatty acyl-CoA oxidase activity in rats treated with EPA and structural analogues of 2-methyl-EPA (all as ethyl esters) for 5 days [a]

Parameters	Compound	Dose (mg/day kg body weight)			
		0	250	600	1500
Peroxisomal β-oxidation (nmol/min mg protein)	EPA	3.35 ± 0.11	3.50 ± 0.21	3.33 ± 0.22	4.36 ± 0.23 [b]
	2-D-EPA	3.07 ± 0.50	3.50 ± 0.52	4.28 ± 0.34 [b]	3.61 ± 0.31
	2,2-diD-EPA	3.02 ± 0.33			4.35 ± 0.41 [b]
	2-methyl-EPA	3.50 ± 0.47	4.58 ± 0.22 [b]	6.60 ± 0.29 [b]	7.55 ± 1.68 [b]
	2-D-2-methyl-EPA	2.85 ± 0.15	4.16 ± 0.70 [b]	4.30 ± 0.41 [b]	7.03 ± 1.42 [b]
Fatty acyl-CoA oxidase (nmol/min mg protein)	EPA	6.18 ± 0.12	6.36 ± 0.55	6.29 ± 0.12	7.99 ± 0.55 [b]
	2-D-EPA	6.15 ± 0.72	6.95 ± 0.22	7.94 ± 0.44 [b]	7.18 ± 0.28
	2,2-diD-EPA	5.92 ± 0.36			7.68 ± 0.28 [b]
	2-methyl-EPA	7.26 ± 0.54	9.22 ± 0.72 [b]	11.53 ± 0.93 [b]	11.94 ± 1.26 [b]
	2-D-2-methyl-EPA	6.52 ± 0.74	8.79 ± 1.20 [b]	9.12 ± 0.55 [b]	12.43 ± 2.07 [b]

[a] The enzyme activities were measured in the light mitochondrial fraction. Values are presented as mean ± SD from 6 animals.
[b] $P < 0.05$ as compared to controls.

given, the serum triglycerides decreased, and at a dose of 600 mg/day kg body weight a 50% reduction of serum triglycerides resulted (Table 2). This methylated *n*-3 fatty acid caused no reduction of serum nor hepatic cholesterol (Table 1). However, it lowered serum HDL-cholesterol (Table 2) and hepatic triglycerides (Table 1). 2-Deuterium-2-methyl EPA reduced both plasma and hepatic triglycerides, in addition to reduced plasma cholesterol and plasma HDL-cholesterol. 2,2-dideuterium EPA reduced plasma triglycerides, cholesterol and HDL-cholesterol.

3.3. Peroxisomal β-oxidation

The activities of peroxisomal β-oxidation and fatty acyl-CoA oxidase were significantly increased (1.3-fold) after administration of EPA at a dose of 1300 mg/day kg body weight (Table 3). Administration of deuterium analogues of EPA stimulated the peroxisomal enzyme activities to a similar magnitude, or even higher, at a dose of 600 mg/day kg body weight (Table 3). Repeated administration of 2-methyl-EPA caused a dose-related increase of the peroxisomal enzyme activities. At the highest dose (1300 mg/day kg body weight), a 2.3-fold increase of peroxisomal β-oxidation and 1.8-fold of fatty acyl-CoA oxidase was revealed (Table 3).

3.4. Platelet aggregation

Aggregation of platelets from rats in the different groups was induced with the divalent cationophore A23187 and adenosine diphosphate (ADP). In platelets from control rats, 1 μM ADP gave a rapid shape change (increase in O.D.) followed by a modest aggregation response (decrease in O.D.) which was reversible with a maximum at 1 min; at 4 μM ADP a greater, reversible aggregation with maximum at 2 min was observed (Fig. 2(A), upper two tracings). In the same control, platelets A23187, at both 2.5 and 5 μM, gave a rapid, transient shape-change response followed by maximal, irreversible aggregation (Fig. 2(A), lower two tracings). Feeding of rats with EPA and its two derivatives had a small, inhibitory effect on platelet aggregation in response to 4 μM ADP, while it caused a marked reduction of the aggregation in response to 1 μM ADP (Fig. 2(B–D)). Similarly, dietary administration of EPA (Fig. 2(B)) and 2-deuterium EPA (Fig. 2(C)) produced a reduction in aggregation induced by the two concentrations of A23187. In sharp contrast, however, administration of dietary 2-methyl EPA actually abolished the aggregatory response to A23187 at both 2.5 and 5 μM,

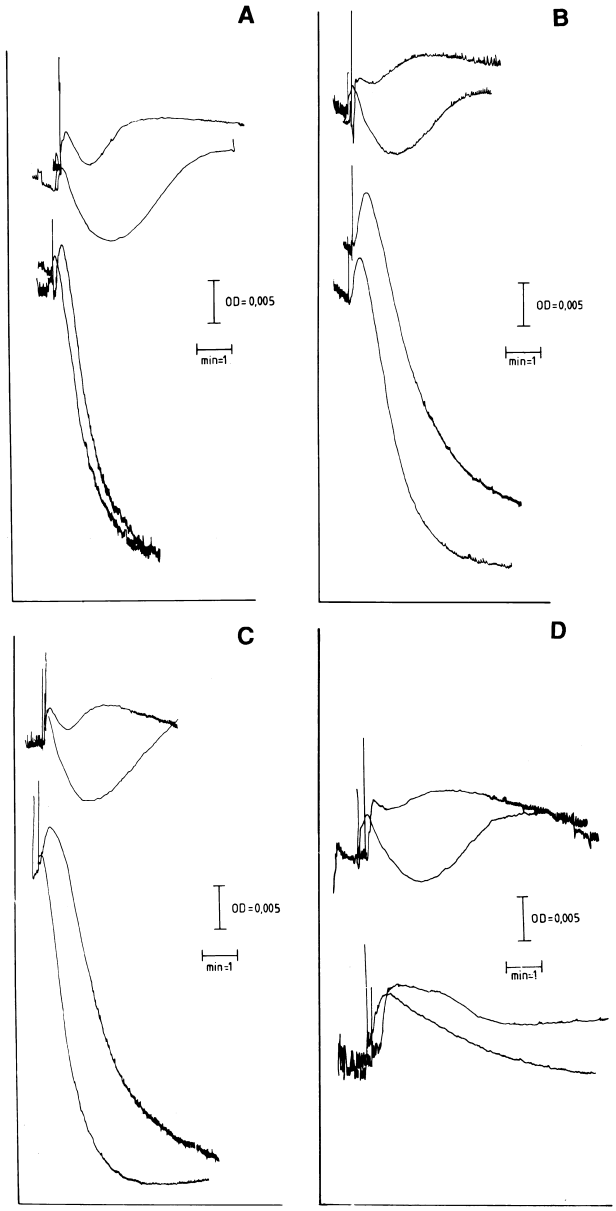

Fig. 2. Platelet aggregation in platelet-rich plasma from rats fed on EPA and its derivatives for 5 days (1300 mg/day kg body weight). Upper two tracings, ADP: 1 μM (trace 1), 4 μM (trace 2). Lower two tracings, A23187: 2.5 μM (trace 3), 5 μM (trace 4). A = control; B = EPA; C = 2-deuterium EPA; and D = 2-methyl EPA. The tracings are representative for three rats in each group.

leaving only a sluggish shape-change response to occur (Fig. 2(D)). Thus, feeding rats with EPA and its derivatives for 5 days diminished platelet aggregation to the low dose of ADP and to both doses of A23187, except that feeding with 2-methyl EPA converted the pronounced and irreversible A23187-induced platelet aggregation response seen in control rats to a faint, reversible shape change response.

Aggregation responses to the two agonists were determined in platelets from rats fed on increasing concentrations of EPA and its derivatives for 5 days (Figs. 3–6). In these dose-response experiments feeding of 1300 mg/kg day of EPA and 2,2-dideuterium EPA for 5 days had no effect on aggregation by 1 μM ADP (Fig. 3). In accordance with the results obtained with 1300 mg/kg day of EPA (Fig. 2), there was also no effect on aggregation produced by 4 μM ADP by administration of 1300 mg/kg day of EPA, and the same result was obtained with 2,2-dideuterium EPA (Fig. 4). However, feeding with 2-

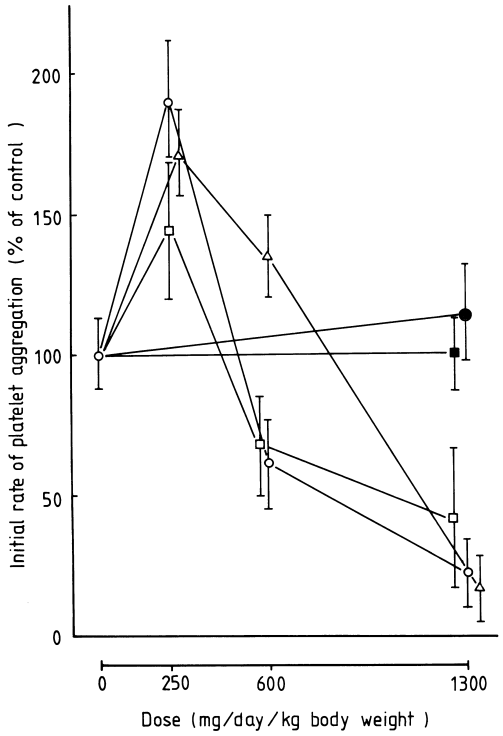

Fig. 3. Dose-dependent changes of the initial rate of platelet aggregation induced by 1 μM ADP in platelet-rich plasma from rats fed EPA (–●–), 2-deuterium-EPA (–○–), 2-methyl-EPA (–□–), 2-deuterium-2-methyl-EPA (–△–) and 2,2-dideuterium-EPA (–■–). Data represent mean ± SD derived from three separate experiments.

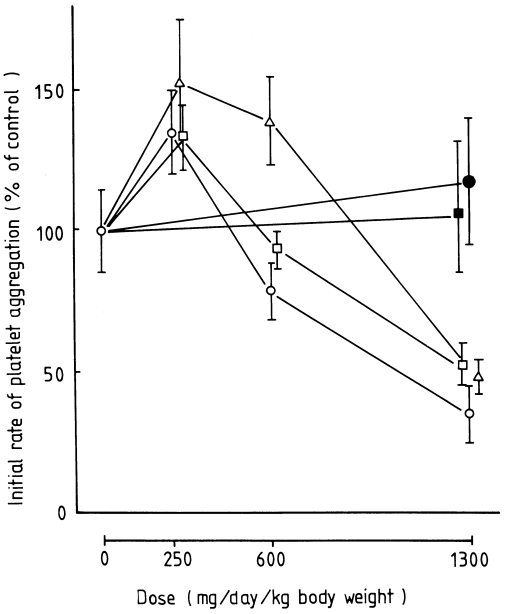

Fig. 4. Dose-dependent changes of the initial rate of platelet aggregation induced by 4 μM ADP in platelet-rich plasma from rats fed EPA (–●–), 2-deuterium-EPA (–○–), 2-methyl-EPA (–□–), 2-deuterium-2-methyl-EPA (–△–) and 2,2-dideuterium-EPA (–■–). Data presents mean ± SD from three separate experiments.

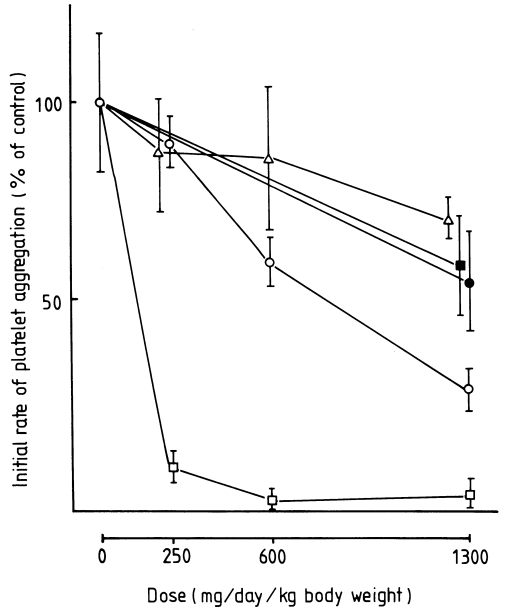

Fig. 5. Dose-dependent changes of the initial rate of platelet aggregation induced by 2,5 μM A 23187 in platelet-rich plasma from rats fed EPA (–●–), 2-deuterium-EPA (–○–), 2-methyl-EPA (–□–), 2-deuterium-2-methyl-EPA (–△–) and 2,2-dideuterium-EPA (–■–). Data are presented as mean ± SD from three separate experiments.

methyl EPA, 2-deuterium EPA and 2-deuterium-2-methyl EPA produced biphasic dose-response curves with an increase in the aggregation response to both doses of ADP at 250 mg/kg day and a progressive inhibition of aggregation as the dose was increased further (Figs. 3 and 4). The biphasic pattern was most expressed for aggregation with 1 μM ADP (Fig. 3). The aggregation response to 2.5 μM A23187 decreased progressively and in parallel with increasing dose of EPA, 2,2-dideuterium EPA and 2-deuterium-2-methyl EPA to ~30% inhibition at 1300 mg/kg day, while 2-deuterium EPA gave a slightly greater, progressive inhibitory effect with 70% inhibition at 1300 mg/kg day (Fig. 5). In sharp contrast, and similar to the observation in Fig. 2(D), 2-methyl EPA inhibited the aggregation response to 2.5 μM A23187 by 90% at 250 mg/kg day and abolished aggregation at higher doses (Fig. 5). The aggregation response to 5 μM A23187 was not affected by EPA and 2,2-dideuterium EPA in the concentration range tested, but gave a biphasic dose-response pattern that was reminiscent of ADP for 2-deuterium EPA and 2-deuterium-2-methyl EPA (Fig. 6). Administration of 2-methyl EPA caused profound inhibition of aggregation with 5 μM A23187 although without abolition at the higher doses (Fig. 6). Thus, administration of EPA and 2,2-dideuterium EPA to the rats were without effect, while the 2-methyl-, 2-deuterium- and 2-deuterium-2-methyl derivatives of EPA had equipotent stimulatory/inhibitory effect on ADP-induced platelet aggregation. For the A23187-induced platelet aggregation, 2-methyl EPA had an outstanding, only-inhibitory effect while EPA and the other derivatives affected aggregation to different degrees.

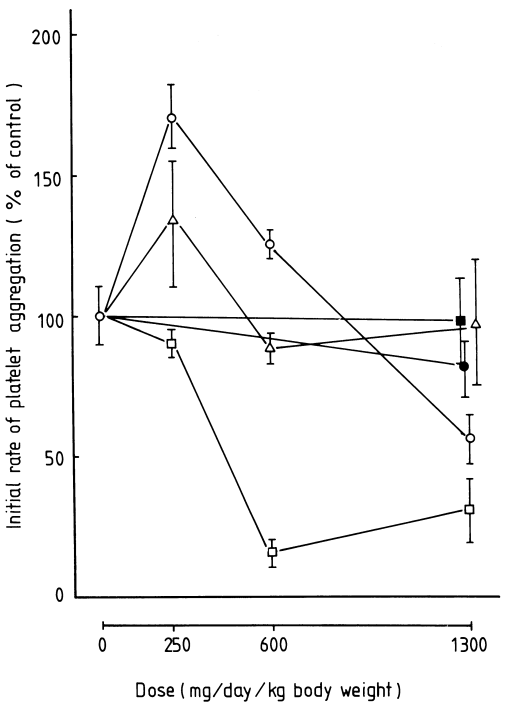

Fig. 6. Dose-dependent changes of the initial rate of platelet aggregation induced by 5 μM A 23187 in platelet-rich plasma from rats fed EPA (– ● –), 2-deuterium-EPA (– ○ –), 2-methyl-EPA (– □ –), 2-deuterium-2-methyl-EPA (– ▲ –) and 2,2-dideuterium-EPA (– ■ –). Data are presented as mean ± SD from three separate experiments.

4. Discussion

3-Thia fatty acids and *n*-3-fatty acids are hypolipidemic substances, i.e. they reduce serum concentrations of cholesterol and triglycerides [15,27], induce proliferation of peroxisomes in rats and have several effects on intermediary lipid metabolism in common. Thus, these hypolipidemic compounds may excert their functions as lipid-lowering agents and peroxisome proliferators, in a similar fashion.

The 3-thia-fatty acid, however, is a fatty acid analogue in which the 3-methylene group of a normal saturated fatty acid is substituted with a sulfur atom. The analogue has many of the biochemical and biophysical properties of a fatty acid [23], but it cannot be β-oxidized in the mitochondria [19]. Thus, it is a paradox that these fatty acid derivatives cannot be themselves oxidized, but can induce hepatic fatty acid β-oxidation and lower plasma triglycerides after administration [15,27].

The correlation coefficient between the specific activities of fatty acyl-CoA oxidase and/or peroxisomal β-oxidation and the volume fraction of peroxisomes, based on our experiments with sulfur-substituted fatty acid analogues, was very good to excellent [19]. Therefore, we suppose that fatty acyl-CoA oxidase activity and peroxisomal β-oxidation are good indicators of peroxisome proliferation.

The present study confirms earlier observations [8,9], that treatment with pure EPA stimulates peroxisomal β-oxidation and fatty acyl-CoA oxidase activity (Table 3). Furthermore, we have found here that

there are clear structural requirements for peroxisomal β-oxidation in rat liver. From the investigation of the structural analogues of EPA (Fig. 1), it can be concluded that the most effective stimulator has a methyl group as the substituent on the carbon 2 of the main chain. This observation is in agreement with data obtained by Lundgren et al. [28], where 2-ethylhexaenoic acid was a more effective peroxisome proliferator than hexanoic acid. Methyl-substituted, non-β-oxidizable long-chain dioic acids have been found to have peroxisome proliferating potential [28].

Based on the results obtained with 3-thiadicarboxylic acid (blocked for ω- and β-oxidation), tetradecylthioacetic acid (blocked for β-oxidation) and tetradecylthiopropionic acid (a ω- and β-oxidizable fatty acid), it is quite evident that availability to β-oxidation is a determinant for peroxisome proliferating effect [18]. Substitution of H with deuterium and CH_3 at carbon 2 of EPA stimulate the peroxisomal β-oxidation and fatty acyl-CoA oxidase activity compared to EPA and 2-methyl-EPA (Table 3). Thus, data obtained with deuterium analogues seem to emphasize the importance of the metabolic availability. The order of potency with respect to stimulation of peroxisomal activities was 2-methyl-EPA = 2-deuterium-2-methyl-EPA > EPA = 2,2-dideuterium-EPA > 2-deuterium-EPA (Table 3). Whether the potency of selected compounds as proliferators of peroxisomes depends on their accessibility for β-oxidation, should be considered. As for the sulfur-substituted fatty acids, the experimental data suggest that the minimal structural requirement for peroxisomal proliferation may be a carboxylic acid group linked to a hydrophobic backbone with poor susceptibility to β-oxidation. The stimulation of peroxisomal activities with EPA and their analogs, but not with palmitic acid [8], conforms to the previously defined requirement for initiation of peroxisomal proliferation [23]. Forman et al. [29] have found hypolipidemic drugs, including tetradecylthioacetic acid, and polyunsaturated fatty acids to be ligands for the peroxisome proliferator-activated receptor α (PPARα), and also they conclude that a carboxyl group on the fatty acid is required for activation of PPARα. As a single fatty acid which is poorly oxidized by mitochondria, EPA has been established as a sufficient stimulus for induction of peroxisomal β-oxidation [9]. The hydrophobicity of the compounds may relate to their accessibility for activation or to their rate of metabolic clearance. As 2-methyl-EPA was a more effective inducer of fatty acyl-CoA oxidase than EPA, poor susceptibility to β-oxidation is, apparently, a determinant of peroxisome proliferating potency.

The present study confirms earlier observations [8,9] that treatment with EPA decreases levels of serum triglycerides (Table 2). In the dose-dependent studies, 2-methyl-EPA was able to reduce serum triglycerides and HDL-cholesterol even more as compared to EPA. Also, substitution of deuterium in the 2-position of the carbon chain tended to reduce the serum lipids. Thus, the order of potency with respect to the lipid lowering effects and peroxisomal β-oxidation, was 2-methyl-EPA = 2-deuterium-2-methyl EPA > 2,2-dideuterium EPA = EPA > 2-deuterium-EPA. 2-Methyl-EPA reduced the liver content of triglycerides (Table 1). Thus, one hypothesis for the triglyceride-lowering effect observed with these polyunsaturated fatty acid analogues is that the effect is due to increased fatty acid oxidation accompanied by a reduction in the availability of fatty acids for triglyceride biosynthesis. EPA can increase β-oxidation in cultured hepatocytes [8] and there is evidence that this pathway is the primary site for the hypolipidemic effect of *n*-3 fatty acids in rats [30–32] as well as in humans [33]. The increase in fatty acid oxidation is able to divert fatty acids from triglyceride synthesis (Table 3) and from the serum compartment [31]. The second hypothesis asserts that the diversion of fatty acids to β-oxidation would also increase the rate of transfer of fatty acids from the plasma into liver, giving a further reduction in plasma fatty acid levels.

Feeding rats 2-methyl- and 2-deuterium-substituted EPA derivatives had profound inhibitory effects on ex vivo platelet aggregation induced by ADP and A23187, particularly at low concentrations of the agonists. Unmodified EPA and 2,2-dideuterium-EPA, however, had no effect in our experiments, except for a 30% inhibition with 1300 mg/kg day at the lowest A23187 concentration. EPA is known to replace arachidonic acid in the platelet phospholipids of rats fed on fish oil [34,35], EPA [36] or EPA precursors [37] without affecting platelet function, which is in accordance with the present results. The cyclooxygenase and lipooxygenase products of EPA liberated from the phospholipids may also counteract platelet

stimulation [38,39]. Thus, replacement of arachidonic acid by EPA in the phospholipids may lower the amount of eicosanoids that promote platelet activation. It is noteworthy that EPA is incorporated in PC and PI and these molecular species may be unsuitable substrates for PLC and PLA_2 which are activated during platelet stimulation [40]. The inhibition of rat platelet aggregation by dietary 2-substituted EPA shown in the present study is, therefore, hardly due to a putative metabolism of these derivatives through the cyclooxygenase pathway. The dose-response experiments revealed a ranking potency order for inhibition, at the highest doses used, of 2-deuterium-EPA = 2-methyl-EPA = 2-deuterium-2-methyl-EPA > EPA = 2,2-dideuterium-EPA for ADP, and 2-methyl-EPA > 2-deuterium-EPA > EPA = 2,2-dideuterium-EPA = 2-deuterium-2-methyl-EPA for A23187 (Figs. 3–6). The most conspicuous similarity among these ranking orders and that for stimulation of peroxisomal activities (above) is that 2-methyl-EPA produces the greatest effect. One reason for this might be that 2-methyl EPA is incorporated into the phospholipids (data to be published). The most outstanding dissimilarity, however, is for 2-deuterium-EPA which, at the highest dose used, did not stimulate peroxisomal activities, but produced maximal inhibition of ADP-induced aggregation and about half-maximal inhibition of A23187-induced aggregation. The different ranking order of the EPA-derivatives to affect platelet aggregation and to cause hypolipidemia suggests different mechanisms.

Inhibition of platelet aggregation by both agonists used by the EPA derivatives was most expressed at the lower agonist concentration. Thus, the inhibition could be overcome by increase of the agonist concentration. For A23187-induced aggregation, the 2-mono-substituted EPA derivatives were more inhibitory than 2-di-substituted EPA at 1300 mg/kg day, and the same pattern is apparent for inhibition of ADP-induced aggregation at 600 mg/kg day. Furthermore, the heavier CH_3-substitution produces more inhibition than the lighter deuterium (D)-substitution. It is expected that substitution of ^1H with CH_3 at the C_2 carbon in EPA would increase the bulkiness around C_2. Feeding the rats with the highest doses of 2-methyl EPA produced the most pronounced inhibition of both ADP- and A23187-induced platelet aggregation. Assuming that substitution with both D and CH_3 at C_2 would also increase bulkiness, and feeding the rats this derivative caused maximal inhibition of ADP-induced aggregation, but had no effect relative to EPA on A23187-induced aggregation. Consequently, our results may indicate that an increase in the bulkiness around C_2 tends to inhibit platelet aggregation. If these 2-methyl-derivatives of EPA were incorporated into the platelet phospholipids, the inhibition of platelet aggregation may have been due to an alteration of acyl-packing of the membrane phospholipids. Indeed, 2-methyl EPA is found in phospholipids in in-vitro experiments (data to be published).

However, substitution of one ^1H with ^2H (D) at C_2 (asymmetric substitution) in EPA would cause a negligible change in bulkiness. Nevertheless, we observed strong inhibition of both ADP- and A23187-induced platelet aggregation after feeding the rats with the highest dose of the asymmetric, 2-D substituted EPA. On the other hand, when the rats were fed with EPA, which had both ^1H-substituted by D at the C_2 carbon (symmetric substitution), no inhibition of platelet aggregation with both agonists relative to non-derivatized EPA was observed. Substitution of one ^1H with CH_3 at C_2 is also a monosubstitution which produced strong inhibition. Our results, therefore, show that feeding with asymmetrically substituted EPA produces inhibition of platelet aggregation ex vivo, while feeding the rats symmetrical substituted EPA does not. The only exception from this apparent rule was our results from feeding rats with the asymmetric 2-deuterium-2-methyl EPA in the experiments with A23187-induced aggregation.

The effect of the dietary 2-substituted EPA was biphasic, i.e. they stimulated and inhibited platelet aggregation at low and high doses, respectively. This phenomenon is known from studies on the effect of amphiphilic cationic drugs (phenothiazines, tricyclic antidepressants) on activation of human platelets with thrombin [41].

Acknowledgements

The authors are grateful to Randi Sandvik, Sissel Rongved and Svein Krüger for excellent technical

assistance. The work was supported by grants from the Norwegian Cancer Society (DNKF), Pronova AS and Bergen Heart Foundation. Special thanks to Morten Lundquist and Bernt Børretsen from Norsk Hydro, Research Centre, Porsgrunn, Norway, who synthesized the EPA derivatives and made these experiments possible.

References

[1] J. Dyerberg, H.O. Bang, N. Hjorne, Am. Clin. Nutr. 28 (1975) 958–966.
[2] A. Hirai, T. Hamasaki, T. Terano, T. Nishikawa, Y. Tamura, A. Kumagai, Lancet 2 (1980) 1132–1133.
[3] D. Kromhout, E.B. Bosschieter, C. de-Lezenne-Coulander, N. Engl. J. Med. 312 (1985) 1205–1209.
[4] S.E. Norell, A. Ahlbom, M. Feychting, N.L. Pedersen, Brit. Med. J. 293 (1986) 426.
[5] S.H. Wong, P.J. Nestel, R.P. Trimble, G.B. Storer, R.J. Illmann, D.L. Topping, Biochim. Biophys. Acta 792 (1984) 103–109.
[6] M.A. Halminski, J.B. March, E.H. Harrison, J. Nutr. 121 (1991) 1554–1561.
[7] M.E. Surette, J. Whelan, G.-P. Lu, K.S. Broughton, J.E. Kinsella, J. Lipid Res. 33 (1992) 263–271.
[8] N. Willumsen, J. Skorve, S. Hexeberg, A.C. Rustan, R.K. Berge, Lipids 28 (1993) 683–690.
[9] A. Aarsland, M. Lundquist, B. Børretsen, R.K. Berge, Lipids 25 (1990) 546–548.
[10] P.J. Nestel, W.E. Connor, M.F. Reardon, S. Connor, S. Wong, R. Boston, J. Clin. Invest. 74 (1984) 82–89.
[11] A.C. Rustan, J.Ø. Nossen, E.N. Christiansen, C.A. Drevon, J. Lipid Res. 29 (1988) 1417–1426.
[12] B.E. Phillipson, D.W. Rothrock, W.E. Connor, W.S. Harris, D.R. Illingworth, N. Engl. J. Med. 312 (1985) 1210–1216.
[13] S.H. Wong, E.A. Fisher, J.B. March, Arteriosclerosis 9 (1989) 836–841.
[14] C.A. Lang, R.A. Davis, J. Lipid Res. 31 (1990) 2079–2086.
[15] W.S. Harris, J. Lipid Res. 30 (1989) 785–807.
[16] P.J. Nestel, Annu. Rev. Nutr. 10 (1990) 149–167.
[17] G. Hornstra, The Significance of Fish and Fish-oil Enriched Food for Prevention and Therapy of Cardiovascular Disease, Academic Press Limited, London, 1989.
[18] A. Aarsland, N. Aarsæther, J. Bremer, R.K. Berge, J. Lipid Res. 30 (1989) 1711–1718.
[19] R.K. Berge, A. Aarsland, H. Kryvi, J. Bremer, N. Aarsæther, Biochem. Pharmacol. 38 (1989) 3969–3979.
[20] H. Singh, K. Beckman, A. Poulos, J. Biol. Chem. 269 (1994) 9514–9520.
[21] P.M. Herold, J.E. Kinsella, Am. J. Clin. Nutr. 43 (1986) 566–598.
[22] S.D. Kristensen, E.B. Schmidt, J. Dyerberg, J. Intern. Med. Suppl. 225 (1989) 141–150.
[23] R.K. Berge, A. Aarsland, H. Kryvi, J. Bremer, N. Aarsæther, Biochim. Biophys. Acta 1004 (1989) 345–356.
[24] R.K. Berge, T. Flatmark, H. Osmundsen, Eur. J. Biochem. 141 (1984) 637–644.
[25] G.M. Small, K. Burdett, M.J. Connock, Biochem. J. 227 (1985) 205–210.
[26] G.P. Mannaerts, L.J. Debeer, J. Thomas, P.J. deSchepper, J. Biol. Chem. 254 (1979) 4585–4595.
[27] G. Gibson, B. Lake,in R.K. Berge, H. Kryvi, N. Aarsæther, A. Aarsland, J.R. Lillehaug, J. Skorve (Eds.), Peroxisomes, Biology and Importance in Toxicology and Medicine, 1993, Chap. 12. pp. 277–297.
[28] B. Lundgren, J. Meijer, J.W. DePierre, Eur. J. Biochem. 163 (1987) 423–431.
[29] B.M. Forman, J. Chen, R.M. Evans, Proc. Natl. Acad. Sci. USA 94 (1997) 4312–4317.
[30] N. Willumsen, S. Hexeberg, J. Skorve, M. Lundquist, R.K. Berge, J. Lipid Res. 34 (1993) 13–22.
[31] N. Willumsen, H. Vaagenes, D. Asiedu, Ø. Lie, A.C. Rustan, R.K. Berge, Lipids 31 (1996) 579–592.
[32] L. Frøyland, L. Madsen, H. Vaagenes, G.K. Totland, J. Auwerx, H. Kryvi, B. Staels, R.K. Berge, J. Lipid Res. 1997, in press.
[33] H. Pownall, CVD/Lipids Dialog, 1997, January, 8–9.
[34] M.Y. Abeywardena, P.L. McLennan, J.S. Charnock, Atherosclerosis 66 (1987) 181–189.
[35] M. Maranesi, V. Barzanti, G. Tarozzi, E. Turchetto, Biochem. Int. 16 (1988) 349–357.
[36] A. Nordøy, E. Davenas, M. Ciavatti, S. Renaud, Biochim. Biophys. Acta 835 (1985) 491–500.
[37] T.W. Weiner, H. Sprecher, Biochim. Biophys. Acta 792 (1984) 293–303.
[38] C. von-Schacky, P.C. Weber, J. Clin. Invest. 76 (1985) 2446–2450.
[39] S. Fischer, P.C. Weber, Biochim. Biophys. Res. Commun. 116 (1983) 1091–1099.
[40] A.M. Bakken, M. Farstad, H. Holmsen, J. Appl. Physiol. 70 (1991) 2669–2672.
[41] P. Tharmapathy, H. Holmsen, Thromb. Haemost. 65 (1991) 1080 (abstract 1387).