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Effect of n-3 fatty acids on serum lipid levels and hepatic fatty acid metabolism in BALB/c.KOR-*Apoe*^{shl} mice deficient in apolipoprotein E expression

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Abstract

N-3 fatty acids exert a potent serum lipid-lowering effect in rodents mainly by affecting hepatic fatty acid oxidation and synthesis. However, it has been observed that fish oil and docosahexaenoic acid ethyl ester do not lower serum lipid levels in apolipoprotein E (apoE)-knockout (*Apoe*^{tm1Unc}) mice generated by gene targeting. To test the hypothesis that apoE expression is required for n-3 fatty acid–dependent regulation of serum lipid levels and hepatic fatty acid metabolism, we examined the effect of fish oil and n-3 fatty acid ethyl esters on the activity and gene expression of hepatic enzymes involved in fatty acid oxidation and synthesis using an alternative apeE-deficient mouse model with the BALB/c genetic background (BALB/c.KOR-*Apoe*^{shl}). ApoE-deficient mice were fed diets containing 9.4% palm oil, fish oil, or 5.4% palm oil and 1% EPA plus 3% DHA ethyl esters for 15 days. In contrast to the reported data on apoE-knockout mice, fish oil and n-3 fatty acid ethyl esters greatly decreased serum triacylglycerol, cholesterol, and phospholipid levels in the *Apoe*^{shl} mice. The decreases were greater with fish oil than with ethyl esters. The alterations by dietary n-3 fatty acids of serum lipid levels were accompanied by parallel changes in the activity and mRNA levels of enzymes involved in hepatic fatty acid oxidation and synthesis. The reason for the discrepancy between the results of the current study and previous studies is unknown. However, our study at least indicates that a lack of apoE expression does not necessarily accompany deficits in the n-3 fatty acid–dependent regulation of serum lipid levels and hepatic fatty acid metabolism.

Keywords: n-3 Fatty acids; Fish oil; Fatty acid oxidation; Fatty acid synthesis; Apolipoprotein E-deficient mice

1. Introduction

It is well recognized that fish oil rich in n-3 fatty acids (eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) exerts a potent serum lipid-lowering effect in mice [1–6] and rats [7–9], and in humans [10,11]. Regarding the mechanism by which fish oil reduces serum lipid levels, it has been demonstrated that fish oil increases the activity and gene expression of hepatic fatty oxidation enzymes in mice [12,13] and rats [7,9]. Studies also demonstrated that fish oil reduces the activity and mRNA levels of enzymes involved in fatty acid synthesis in mice [4,12] and rats [7,8]. The up-regulation of hepatic fatty acid oxidation and the down-regulation of lipogenseis may decrease the availability of fatty acids for synthesizing triacylglycerol in liver and in turn may affect the production and secretion of triacylglycerol-rich, very low density lipoprotein. This would account for the serum lipid lowering effect of fish oil. Several studies showed that purified EPA and DHA in the form of ethyl esters also caused a reduction in serum lipid levels accompanying a change in hepatic fatty acid metabolism in rats [14–17].

Apolipoprotein E (apoE) is an important constituent of triacylglycerol-rich very low density lipoprotein and chyromicorn, and an important ligand for lipoprotein receptors in tissues [18], and hence plays a crucial role in regulating serum lipid levels. In this context, it has been well demonstrated that apoE polymorphisms strongly influences cholesterol and triacylglycerol metabolism in humans [19,20].

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A deficiency of apoE in mice results in a retardation of triacylglycerol-rich lipoprotein clearance and accumulation of remnant-like particles in the blood stream [21–23]. In this regard, apoE-deficient mice would serve as a suitable animal model of hyperlipidemia for the assessment of the physiological activity of dietary factors such as fish oil that affect hepatic lipoprotein production. Unexpectedly, however, Asset et al. [2] showed that fish oil, compared to coconut oil, is totally ineffective in reducing plasma cholesterol levels in male apoE-knockout (*Apoe*^{tm1Unc}) mice. Compared to both sunflower and coconut oils, fish oil caused more than a 4-fold increase in plasma triacylglycerol concentrations in the apoE-knockout mice despite that it decreased plasma cholesterol and triacylglycerol concentrations in the wild-type mice. Adan et al. [24] showed that DHA ethyl ester caused a slight decrease in serum cholesterol levels in female apoE-knockout mice. However, it was ineffective in reducing this parameter in male apoE-knockout mice. Also, DHA ethyl ester did not affect serum triacylglycerol concentrations in female apoE-knockout mice and approximately doubled the levels in male apoE-knockout mice. These observations may go against the general notion that the reduction in hepatic lipoprotein production through the alteration of fatty acid oxidation and synthesis is a crucial factor accounting for the physiological activity of n-3 polyunsaturated fatty acids in reducing serum lipid levels.

We recently established a line of mice derived from a KOR inbred strain of Japanese wild mice deficient in the expression of apoE (KOR-*Apoe*^{shl}) due to a gross disruption of the gene for this protein [22]. The mice show hypercholesterolemia and accumulate huge amounts of remnant-like particles in the blood stream as has been observed in apoE-knockout (*Apoe*^{tm1Unc}) mice. However, the original KOR-*Apoe*^{shl}mice were found to be inappropriate for use in nutritional studies because they do not eat purified experimental diets and die several days after their diet is changed from laboratory chow (Ide et al., unpublished observation). Therefore, we have developed several congenic apoE-deficient mice with the genetic background of laboratory mice by transferring the apoE gene mutation from the KOR genetic background through repeated back-crossing [23]. An alternative apoE-deficient murine model to the *Apoe*^{tm1Unc} mouse may be useful for confirming the role of apoE in the nutritional regulation of lipid metabolism.

Previous findings [2,24] that dietary n-3 fatty acids are rather irrelevant in reducing serum lipid levels in apoE-knockout (*Apoe*^{tm1Unc}) mice possibly indicated that apoE expression is required for n-3 fatty acid–dependent regulation of hepatic fatty acid metabolism or the basal metabolic activities of fatty acid oxidation and synthesis are too low in apoE-deficient mice to affect hepatic lipoprotein production. However, a study to examine the effect of n-3 fatty acids on hepatic fatty acid oxidation and synthesis in apoE-deficient mice has been lacking. In the current study we used congenic mice with a BALB/c genetic background (BALB/c.KOR-*Apoe*^{shl}) as an alternative animal model of apoE-deficiency to examine the effects of fish oil and n-3 fatty acid ethyl esters on serum lipid levels as well as the activity and mRNA levels of enzymes involved in hepatic fatty acid oxidation and synthesis. This study was conducted to examine whether the deficits in the n-3 fatty acid–dependent regulation of serum lipid levels are a general consequence of a deficiency in apoE expression in mice.

2. Methods and materials

2.1. Animals and diets

In the first experiment, we compared serum and liver lipid levels as well as the activity for hepatic fatty acid metabolism between normolipidemic BALB/c mice and hyperlipidemic BALB/c.KOR-*Apoe*^{shl} mice deficient in apoE expression. Five male normolipidemic mice and seven male apoE-deficient mice at 5–7 weeks of age were fed an experimental diet containing 10% palm oil for 14 days. The average body weight at the start of the experiment was slightly lower in normolipidemic (18.0 ± 0.6 g) than in hyperlipidemic mice (20.3 ± 1.3 g), but the difference was not significant. Animals had free access to the diets and water during the experimental period. In the second trial, we examined the effect of fish oil and n-3 fatty acid ethyl esters on serum and liver lipid levels, and the activity and gene expression of hepatic enzymes involved in fatty acid oxidation and synthesis in hyperlipidemic BALB/c.KOR-*Apoe*^{shl} mice. Male and female hyperlipidemic BALB/c.KOR-*Apoe*^{shl} mice at the age of 6–11 weeks were randomly divided into three groups consisting of 11 animals (five to six males and females in each group) and fed experimental diets containing 9.4% palm oil, fish oil, or 5.4% palm oil and 1% EPA plus 3% DHA ethyl esters. The average body weight of mice at the start of the second experiment was 24.6 ± 1.0, 23.1 ± 1.2, and 23.2 ± 0.9 g for the palm oil, EPA+DHA, and fish oil groups, respectively, and all differences were nonsignificant. Animals had free access to the diets and water during the experimental period as in the first experiment. We followed our institute's guidelines in the care and use of laboratory animals. The basal composition of the purified experimental diet was detailed elsewhere [7]. Palm and fish oils were gifts from Nippon Oil & Fats Co. (Tokyo, Japan). DHA and EPA ethyl esters were donated by Harima Chemicals (Tsukuba, Japan) and Kewpie Co. (Tokyo, Japan), respectively. The fatty acid compositions of diets and sum of the values for the amounts of n-3 fatty acids in 100 g of each diet used in the second experiment are shown in Table 1. The palm oil diet was almost free of n-3 fatty acids. Other diets contained equivalent amounts of n-3 fatty acids mainly as EPA and DHA.

Table 1
Fatty acid composition of diets

	Diet		
	9.4% Palm oil	5.4% Palm oil and 1% EPA plus 3% DHA ethylesters	9.4% Fish oil
Fatty acid (g/100 g total fatty acids)			
14:0	0.87	0.58	3.25
16:0	44.1	25.8	14.0
16:1(n-7)	0.03	0.02	7.88
18:0	4.19	2.44	2.59
18:1(n-9)	41.3	23.7	21.6
18:2(n-6)	9.19	5.28	2.11
18:3(n-3)	0.31	0.19	1.53
20:3(n-6)	0.00	0.10	0.09
20:4(n-6)	0.00	0.04	3.06
20:5(n-3)	0.00	11.2	10.7
22:4(n-6)	0.00	0.00	0.00
22:5(n-6)	0.00	0.01	1.44
22:5(n-3)	0.00	0.02	1.67
22:6(n-3)	0.00	30.7	30.1
n-3 fatty acids (g/100 g diet)	0.03	3.95	4.13

2.2. Enzyme assays

Upon termination of the experiments, animals were anesthetized using diethyl ether and killed by bleeding from the inferior vena cava, after which livers were quickly excised. Liver (approximately 0.8 g) was homogenized with 10 volumes of 0.25 mol/L sucrose containing 1 mmol/L EDTA and 3 mmol/L Tris-HCl (pH 7.2) and centrifuged at 200,000 $\times$ g for 30 minutes. The rate of peroxisomal oxidation of palmitoyl-CoA was measured using total liver homogenates according to the method of Mannaerts et al. [25]. The assay mixture (final volume 2.5 mL) contained 4 mmol/L ATP, 0.5 mmol/L CoA, 2 mmol/L NAD, 2 mmol/L dithiothreitol, 2 mmol/L KCN, 0.2 mmol/L [1-^{14}C] palmitoyl-CoA (0.2 μCi/μmol), and 0.1 mL of the total homogenate in modified Krebs-Henseleit bicarbonate buffer (pH 7.4) [25]. After 5 minutes of incubation at 37°C in Erlenmeyer flasks, the enzyme reaction was terminated by pouring the mixture into test tubes containing 0.625 mL of 30% perchloric acid and acid-soluble radioactivity was measured by liquid scintillation counting. The activity of various enzymes involved in the fatty acid oxidation pathway was measured spectrophotometrically using the whole liver homogenate as an enzyme source. Acyl-CoA oxidase activity as the rate of palmitoyl-CoA–dependent formation of hydrogen peroxide was measured according to the methods of Osumi and Hashimoto [26]. The carnitine-dependent CoA release from palmitoyl-CoA was analyzed to measure the activity of carnitine palmitoyltransferase [27]. The rate of crotonyl-CoA–dependent reduction of NAD in the presence of bovine 3-hydroxyacyl-CoA dehydrogenase (Sigma-Aldrich Co., St. Louis, MO.) represented enoyl-CoA hydratase activity [28]. The rate of acetoacetyl-CoA-dependent oxidation of NADH was taken as the activity of 3-hydroxyacyl-CoA dehydrogenase [29]. 3-Keotacyl-CoA thiolase activity represented the rate of CoA-dependent cleavage of acetoacetyl-CoA [30]. The activity of lipogenic enzymes was measured spectrophotometrically using the 200,000 $\times$ g supernatant fraction of the liver homogenate. Fatty acid synthase activity was measured as malonyl-CoA–dependent oxidation of NADPH in the presence of acetyl-CoA [31]. ATP-citrate lyase activity represented the rate of CoA-dependent oxidation of NADH in the presence of citrate, ATP, and malate dehydrogenase (Oriental Yeast Co., Osaka, Japan) [32]. The rate of NADP reduction after the addition of malic acid was analyzed to measure malic enzyme activity [33]. The rate of glucose 6-phosphate–dependent reduction of NADP in the presence of excess amounts of 6-phosphogluconate dehydrogenase (Oriental Yeast Co., Osaka, Japan) [34] represented glucose 6-phosphate dehydrogenase activity. Pyruvate kinase activity was measured as the rate of phosphoenolpyruvate-dependent oxidation of NADH in the presence of ADP and lactate dehydrogenase (Roche Diagnostics GmbH, Manheim, Germany) [35].

2.3. Lipid analysis

Liver lipid was extracted according to the method of Folch et al. [36] and triacylglycerol [37], phospholipid [37], and cholesterol [38] concentrations in extracts were determined as described before. Hepatic fatty acid contents and compositions were analyzed by gas–liquid chromatography [37] using heptadecanoic acid as an internal standard. Serum triacylglycerol, cholesterol, and phospholipid concentrations were measured using commercial enzyme kits (Wako Pure Chemical, Osaka, Japan).

2.4. RNA analysis

Hepatic RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method [39]. RNA samples (30 μg) were denatured and applied to a nylon membrane using a slot-blot apparatus (Bio-Rad Laboratories, Hercules, CA) and fixed with UV irradiation. Northern blot analysis was conducted by standard procedures. RNA samples (30 μg) were denatured and electrophoresed on a 1.1% agarose gel containing 0.66 mol/L formaldehyde and were then transferred to a nylon membrane and fixed with ultraviolet irradiation. RNAs on nylon membranes were hybridized with specific cDNA probes [7] labeled with [α-^{32}P]dCTP and mRNA levels were quantified with an imaging analyzer (Bio-Rad Laboratories, Hercules, CA).

2.5. Statistical analysis

In the first experiment, significant differences between means were evaluated with the Student t test at the level of $P < 0.05$. For the second experiment the data were analyzed using a one-way analysis of variance, and all significant

Table 2
Activity of enzymes involved in hepatic fatty acid oxidation and synthesis in wild-type (BALB/c) and apoE-deficient (BALB/c.KOR-*Apoe*^{shl}) mice fed a 10% palm oil diet*

Enzyme activity (μmol/min per liver per 100 g body weight)	Animals	
	Wild-type	ApoE-deficient
Enzymes in fatty acid oxidation		
Peroxisomal palmitoyl-CoA oxidation	3.05 ± 0.09	2.75 ± 0.18
Acyl-CoA oxidase	2.26 ± 0.25	1.77 ± 0.29
3-Hydroxyacyl-CoA dehydrogenase	476 ± 13	495 ± 33
3-Ketoacyl-CoA thiolase	177 ± 9	173 ± 8
Enzymes in fatty acid synthesis		
Fatty acid synthase	18.5 ± 1.0	11.7 ± 0.9†
ATP-citrate lyase	24.7 ± 2.2	14.5 ± 1.6†
Malic enzyme	187 ± 12	120 ± 8†
Glucose 6-phosphate dehydrogenase	8.36 ± 1.53	4.88 ± 0.36†
Pyruvate kinase	261 ± 12	198 ± 9†

* Male wild-type BALB/c mice and BALB/c.KOR-*Apoe*^{shl} mice deficient in apoE expression were fed a purified experimental diet containing 10% palm oil for 14 days.

Values are means ± SEM, $n = 5$ and $n = 7$/group for the wild-type and apoE-deficient mice, respectively.

† Values are significantly different from those in the wild-type mice at $P < 0.05$.

differences among means at the level of $P< 0.05$ were inspected with a Tukey-Kramer post hoc analysis [40].

3. Results

3.1. Activity of enzymes involved in hepatic fatty acid oxidation and synthesis, and serum and hepatic lipid levels in wild-type and apoE-deficient mice

In the first experiment, BALB/c mice and hyperlipidemic BALB/c.KOR-*Apoe*^{shl} mice deficient in apoE expression were fed a purified experimental diet containing 10% palm oil for 14 days to clarify the impact of the apoE deficiency on serum and liver lipid levels as well as activity of enzymes involved in hepatic fatty acid metabolism. No significant differences were seen in food intake (3.6 ± 0.1 and 3.6 ± 0.1 g/day for the wild-type and apoE-deficient mice), body weight at time of sacrifice (26.0 ± 0.5 and 27.5 ± 1.1 g, respectively), and liver weight (6.4 ± 0.1 and 6.6 ± 0.1 g/100 g body weight, respectively) between the wild-type and apoE-deficient mice.

Activities of enzymes involved in hepatic fatty acid oxidation and synthesis are summarized in Table 2. The peroxisomal fatty acid oxidation rate and the activity of acyl-CoA oxidase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase were the same between the wild-type and apoE-deficient mice. Activity levels of lipogenic enzymes were reduced in apoE-deficient compared to wild-type mice as follows: fatty acid synthase 37%, ATP-citrate lyase 41%, malic enzyme 36%, glucose 6-phosphate dehydrogenase 42%, and pyruvate kinase 24%, respectively.

Table 3
Serum and liver lipid levels of the wild-type (BALB/c) and apoE-deficient(BALB/c.KOR-*Apoe*^{shl}) mice fed a 10% palm oil diet*

	Animals	
	Wild-type	ApoE-deficient
Serum lipid level (μmol/dL)		
Cholesterol	361 ± 16	2229 ± 8†
Triacylglycerol	178 ± 10	628 ± 39†
Phospholipid	402 ± 7	708 ± 30†
Hepatic lipid content (μmol/liver per 100 g body weight)		
Cholesterol	51.9 ± 2.6	88.7 ± 7.2†
Triacylglycerol	306 ± 30	554 ± 58†
Phospholipid	184 ± 5	194 ± 5

* The experiment was as described in Table 2.

† Values are significantly different from those in the wild-type mice at $P < 0.05$.

ApoE-deficiency resulted in huge increases in serum cholesterol and triacylglycerol levels (Table 3). These levels were 6.2 and 3.5 times higher, respectively, in apoE-deficient mice than in the wild-type mice. Although the increase was attenuated, the serum phospholipid level was also 1.8 times higher in the former than in the latter. Hepatic cholesterol and triacylglycerol levels were 1.7 and 1.8 times higher, respectively, in apoE-deficient mice than in the wild type-mice. However, apoE-deficiency did not affect hepatic phospholipid content.

3.2. Activity and mRNA levels of enzymes involved in hepatic fatty acid oxidation and synthesis, and serum and hepatic lipid levels in apoE-deficient mice fed n-3 fatty acids.

Food intake (3.6 ± 0.1, 3.3 ± 0.1, and 3.5 ± 0.1 g/day for the palm oil, EPA+DHA and fish oil groups, respectively) and body weight at time of sacrifice (26.5 ± 1.2, 25.2 ± 0.8, and 27.4 ± 0.9 g for the palm oil, EPA+DHA and fish oil groups, respectively) among the groups of apoE-deficient mice fed different diets were indistinguishable. Liver weights were significantly higher in mice fed the 9.4% fish oil diet (7.50 ± 0.17 g/100 g body weight) than 9.4% palm oil diet (5.94 ± 0.18 g/100 g body weight), and a diet containing 5.4% palm oil and 1% EPA plus 3% DHA ethyl esters (5.76 ± 0.17 g/100 g body weight).

No sex-dependent differences in the enzyme activity for hepatic fatty acid metabolism were observed; therefore, values for the enzymic activity of male and female mice were combined and analyzed statistically. The diets containing EPA and DHA either in the form of ethyl esters or fish oil compared to the palm oil diet significantly increased the activity of enzymes involved in hepatic fatty acid oxidation (Table 4). The extent of the increase was much weaker in mice given the ethyl ester diet than those fed fish oil as follows: peroxisomal palmitoyl-CoA oxidation 1.6- and 4.6-fold for mice given ethylesters and fish oil, respec-

Table 4
The activity and mRNA levels of enzymes involved in hepatic fatty acid oxidation, and mRNA level of cytochrome P-450 IV A1 in apoE-deficient mice (BALB/c.KOR-*Apoe*^{shl}) fed n-3 fatty acids in the form of EPA and DHA ethyl esters or fish oil*

	Diet		
	9.4% Palm oil	5.4% Palm oil and 1% EPA plus 3% DHA ethyl esters	9.4% Fish oil
Enzyme activity (μmol/min per liver per 100 g body weight)			
Peroxisomal palmitoyl-CoA oxidation	3.69 ± 0.19†	5.89 ± 0.43‡	17.2 ± 0.4§
Acyl-CoA oxidase	1.36 ± 0.10†	2.21 ± 0.12‡	7.72 ± 0.30§
Carnitine palmitoyltransferase	7.91 ± 0.59†	13.0 ± 0.5‡	27.3 ± 0.7§
Enoyl-CoA hydratase	847 ± 58†	1282 ± 79‡	2788 ± 97§
3-Hydroxyacyl-CoA dehydrogenase	472 ± 21†	598 ± 29‡	1039 ± 17§
3-Ketoacyl-CoA thiolase	216 ± 11†	294 ± 20‡	448 ± 18§
mRNA level (%)			
Peroxisomal enzymes			
Acyl-CoA oxidase	100 ± 3†	135 ± 7†	209 ± 23‡
Bifunctional enzyme	100 ± 10†	196 ± 26‡	678 ± 47§
3-Ketoacyl-CoA thiolase	100 ± 5†	144 ± 15†	224 ± 32‡
Mitochondrial enzymes			
Carnitine palmitoyltransferase II	100 ± 4†	143 ± 10‡	204 ± 23§
Trifunctional enzyme			
Subunit α	100 ± 3†	131 ± 10†	208 ± 26‡
Subunit β	100 ± 5†	158 ± 13‡	294 ± 29§
3-Ketoacyl-CoA thiolase	100 ± 3†	141 ± 9‡	199 ± 22§
3-Hydroxy-3-methylglutaryl-CoA synthase	100 ± 4†	153 ± 11‡	186 ± 23†
Cytochrome P-450 IV A1	100 ± 7†	178 ± 21‡	346 ± 37§

* Male and female hyperlipidemic mice deficient in apoE expression (BALB/c.KOR-*Apoe*^{shl}) were randomly divided into 3 groups and fed experimental diets containing 9.4% palm oil, fish oil or 5.4% palm oil and 1% EPA plus 3% DHA ethyl esters for 15 days.

Values are means ± SEM, $n = 11$/group (5–6 males and females).

$^{\dagger, \ddagger, \S}$ Values not sharing the same superscript are significantly different at $P < 0.05$.

tively; acyl-CoA oxidase 1.6- and 5.7-fold; carnitine palmitoyltransferase 1.6- and 3.5-fold; enoyl-CoA hydratase 1.5- and 3.3-fold; 3-hydroxyacyl-CoA dehydrogenase 1.3- and 2.2-fold; and 3-ketoacyl-CoA thiolase 1.4- and 2.1-fold.

Table 4 also shows mRNA abundances of peroxisomal and mitochondrial fatty acid oxidation enzymes in the liver measured by slot-blot hybridization. The values were corrected for those of a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase), and are expressed as percentages assigning the value in animals fed the 9.4% palm oil diet as 100. Fish oil compared to palm oil significantly increased mRNA levels of hepatic peroxisomal and mitochondrial fatty acid oxidation enzymes. The fish oil diet compared to the palm oil diet caused more than a 2-fold increase in mRNA levels of peroxisomal enzymes (2.1-, 6.8-, and 2.2-fold increase for acyl-CoA oxidase, bifunctional enzyme and 3-ketoacyl-CoA thiolase, respectively). The fish oil diet also caused a great increase in the gene expression of various mitochondrial enzymes (carnitine palmitoyltransferase II 2.0-fold, trifunctional enzyme subunit α and β 2.1- and 2.9-fold, 3-ketoacyl-CoA thiolase 2.0-fold, and 3-hydroxy-3-methylglutaryl-CoA synthase 1.9-fold, respectively).. The diets containing EPA and DHA ethyl esters also increased these parameters, but the extent of the increase for both peroxisomal enzymes (acyl-CoA oxidase 1.3-fold, bifunctional enzyme 2.0-fold, and 3-ketoacyl-CoA thiolase 1.4-fold, respectively) and mitochondrial enzymes (carnitine palmitoyltransferase II 1.4-fold, trifunctional enzyme subunit α and β 1.3- and 1.6-fold, 3-ketoacyl-CoA thiolase 1.4-fold, and 3-hydroxy-3-methylglutaryl-CoA synthase 1.5-fold, respectively) was less than that obtained with fish oil, and significant differences compared to the palm oil diet were not confirmed on many occasions. In addition to mRNA levels of hepatic β-oxidation enzymes, the mRNA level of microsomal cytochrome P-450 IV A1 involved in ω-oxidation of fatty acids [41] was also increased by diets containing n-3 fatty acids. Again, the increase was greater in mice fed fish oil (3.5-fold) than ethyl esters (1.8-fold).

The diet containing EPA and DHA ethyl esters and fish oil, compared to the palm oil diet, greatly decreased the activity of lipogenic enzymes in the liver (Table 5). The extent of the decrease was approximately the same between the animals fed ethylesters and fish oil as follows: fatty acid synthase 66.6% and 61.2% for mice given ethylesters and fish oil, respectively, ATP-citrate lyase 68.4% and 77.1%, malic enzyme 51.8% and 33.6%, glucose 6-phosphate dehydrogenase 54.2% and 49.0%, and pyruvate kinase; 36.7% and 49.4%. All the diets containing EPA and DHA either in the form of ethyl esters or fish oil also significantly reduced the gene expression of lipogenic enzymes and sterol regulatory element binding protein-1. The extent of the decrease

Table 5
The activity and mRNA levels of enzymes involved in hepatic fatty acid synthesis, and mRNA level of sterol regulatory element binding protein-lin apoE-deficient mice (BALB/c.KOR-*Apoe*^{shl}) fed n-3 fatty acids in the form of EPA and DHA ethyl esters or fish oil*

	Group		
	9.4% Palm oil	5.4% Palm oil and 1% EPA plus 3% DHA ethyl esters	9.4% Fish oil
Enzyme activity (μmol/min per liver per 100 g body weight)			
Fatty acid synthase	17.3 ± 1.9‡	5.78 ± 0.85†	6.71 ± 0.44†
ATP-citrate lyase	16.5 ± 2.3‡	5.20 ± 0.91†	3.77 ± 0.29†
Malic enzyme	96.7 ± 6.7‡	46.6 ± 5.7†	64.2 ± 2.7†
Glucose 6-phosphate dehydrogenase	7.01 ± 0.99‡	3.21 ± 0.30†	3.58 ± 0.12†
Pyruvate kinase	146 ± 7§	92.7 ± 6.3‡	74.0 ± 3.9†
mRNA level (%)			
Acetyl-CoA carboxylase	100 ± 5‡	62.6 ± 2.2†	60.5 ± 2.7†
Fatty acid synthase	100 ± 10‡	16.1 ± 2.0†	12.3 ± 1.8†
ATP-citrate lyase	100 ± 5‡	57.9 ± 1.0†	54.2 ± 3.1†
Malic enzyme	100 ± 4‡	59.9 ± 2.1†	56.9 ± 1.1†
Pyruvate kinase	100 ± 7‡	39.4 ± 5.9†	26.4 ± 3.5†
Sterol regulatory element binding protein-1	100 ± 10†	64.3 ± 2.5†	55.7 ± 3.0†

* The experiment was as described in Table 4.
Values are means ± SEM, n = 11/group (5-6 males and females).
$^{†, ‡, §}$ Values not sharing the same superscript are significantly different at $P < 0.05$.

was comparable between the animals fed a diet containing EPA and DHA in the form of ethyl esters and fish oil as follows: acetyl-CoA carboxylase 37.4% and 39.5% for mice given ethylesters and fish oil respectively, fatty acid synthase 83.9% and 87.7%, ATP-citrate lyase 42.1% and 45.8%, malic enzyme 40.1% and 43.1%, pyruvate kinase 60.6% and 73.6%, and sterol regulatory element binding protein-1 35.7% and 44.3%.

Northern blot analysis of typical samples of hepatic RNA in apoE-deficient mice fed palm oil, EPA+DHA ethylester and fish oil diets (Fig. 1) confirmed the integrity of RNA samples and specificity of our cDNA probes. In addition, it was apparent that diets containing EPA and DHA either in the form of ethyl esters or fish oil, especially the latter, compared to the palm oil diet, strongly increased mRNA levels of peroxisomal and mitochondrial fatty acid oxidation enzymes and microsomal cytochrome P-450 IV A1 (Fig. 1 A). Also, it was confirmed that EPA+DHA ethylester and fish oil diets reduced mRNA levels of lipogenic enzymes and sterol regulatory element binding protein-1 to similar levels (Fig. 1 B).

The diets containing n-3 fatty acids either in the form of ethyl esters or fish oil greatly decreased serum concentrations of cholesterol, triacylglycerol, and phospholipid in apoE-deficient mice (Table 6). The extent of the decrease in apoE-deficient mice fed fish oil was 83.8%, 75.4%, and 71.1% for cholesterol, triacylglycerol, and phospholipid, respectively. Cholesterol and triacylglycerol levels in apoE-deficient mice fed fish oil almost reached those observed in the wild-type mice fed a 10% palm oil diet, and the phospholipid concentration became even lower than that observed in the wild-type mice (Table 3). Although the extent of the reduction was attenuated, a diet containing n-3 fatty acid ethyl esters, compared to the palm oil diet, also lowered the serum lipid levels as follows: cholesterol 69.4%, triacylglycerol 47.7%, and phospholipid 63.3%. The diets containing n-3 fatty acids also lowered the hepatic amounts of cholesterol and triacylglycerol, the extent of the reduction being comparable with that for ethyl ester and fish oil diets (cholesterol 51.8% and 40.3%, and triacylglycerol; 67.6%

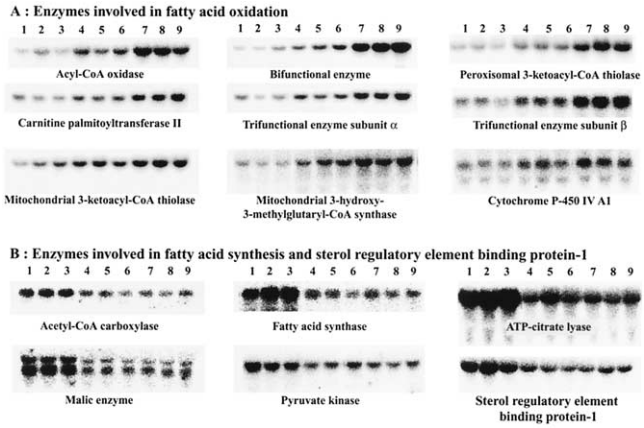

Fig. 1. Northern blot analysis of mRNAs for fatty acid oxidation enzymes (*A*), and lipogenic enzymes and sterol regulatory element binding protein-1 (*B*) in the liver of apoE-deficient mice. RNA samples (30 μg) were denatured and subjected to electrophoresis on 1.1% agarose gel containing 0.66 mol/L formaldehyde, then transferred to a nylon membrane and fixed with UV irradiation. The RNA on nylon membranes was hybridized with radiolabeled cDNA probes specific for mRNAs of respective proteins. Lanes 1–3: Mice fed a 9.4% palm oil diet; lanes 4–6: mice fed a 5.4 % palm oil diet containing 1% EPA plus 3% DHA ethyl esters; lanes 7–9: mice fed a 9.4% fish oil diet.

Table 6
Serum and liver lipid levels in apoE-deficient mice (BALB/c.KOR-*Apoe*^{shl}) fed n-3 fatty acids in the form of EPA and DHA ethyl esters or fish oil*

	group		
	9.4% Palm oil	5.4% Palm oil and 1% EPA plus 3% DHA ethyl esters	9.4% Fish oil
Serum lipid level (μmol/dL)			
Cholesterol	2589 ± 101§	793 ± 90‡	420 ± 37†
Triacylglycerol	839 ± 131§	439 ± 17‡	206 ± 14†
Phospholipid	878 ± 63‡	322 ± 21†	254 ± 7†
Hepatic lipid content (μmol/liver per 100 g body weight)			
Cholesterol	97.6 ± 9.6‡	47.0 ± 1.9†	58.2 ± 1.7†
Triacylglycerol	486 ± 62‡	157 ± 24†	190 ± 22†
Phospholipid	231 ± 8†	328 ± 13‡	366 ± 7§
Hepatic fatty acid content (μmol/liver per 100 g body weight)			
20:5 (n-3)	0.0 ± 0.0†	57.6 ± 3.9‡	60.1 ± 2.6‡
22:5 (n-3)	1.2 ± 0.1†	16.9 ± 0.9‡	34.0 ± 3.1§
22:6 (n-3)	46.1 ± 1.6†	288 ± 14.7‡	406 ± 20§
Sum	47.2 ± 1.7†	362 ± 19‡	501 ± 25§

* The experiment was as described in Table 4.
Values are means ± SEM, $n = 11$/group (5-6 males and females).
$^{\dagger, \ddagger, \S}$ Values not sharing the same superscript are significantly different at $P < 0.05$.

and 60.9% for mice given ethylesters and fish oil, respectively). The diet containing ethyl esters of EPA and DHA and the fish oil diet, compared to the palm oil diet, significantly increased hepatic phospholipid content. Also, this value was significantly higher in mice fed fish oil than in the animals fed ethylesters. Diets containing n-3 fatty acids greatly increased the amount of EPA, docasapentaenoic acid (DPA) and DHA in hepatic lipid in apoE-deficient mice. The sum of the values of these n-3 fatty acids was 1.4-fold higher in the fish oil group than in the ethyl ester group.

4. Discussion

We hypothesized that apoE expression is required for n-3 fatty acid–dependent regulation of hepatic fatty acid metabolism, and a disturbance in the regulation of the metabolic pathway may account for the failure of fish oil to reduce serum lipid levels in apoE-knockout (*Apoe*^{tm1Unc}) mice observed in previous studies [2,24]. To address this point, we used an alternative apoE-deficient model (BALB/c.KOR-*Apoe*^{shl} mice) that we have developed [22,23].

In contrast to previous observations [2,24], fish oil and n-3 fatty acid ethyl esters caused great decreases in serum cholesterol, triacyglycerol, and phospholipid levels in apoE-deficent mice in the present study. The levels in the animals fed fish oil and n-3 fatty acid ethyl esters dropped to less than 30% and 50%, respectively, of the values in the animals fed palm oil. Therefore, the apoE-deficient mice used in the current study in contrast to apoE-knockout mice used by others [2,24] may represent a more sensitive animal model for clarifying the lipid-lowering effect of n-3 fatty acids. We found that the activity of enzymes involved in hepatic fatty acid synthesis but not those involved in fatty acid oxidation was significantly lower in BALB/c.KOR-*Apoe*^{shl} mice than in the wild-type mice. This may account for the lower rate of production of hepatic very low density lipoprotein in apoE-knockout (*Apoe*^{tm1Unc}) mice [42]. However, we also demonstrated that the activity and mRNA levels of enzymes involved in fatty acid oxidation and synthesis in the apoE-deficient BALB/c.KOR-*Apoe*^{shl} mice were regulated by fish oil and n-3 fatty acid ethyl esters in a manner that has been well demonstrated in the wild-type mice [4,12,13] and rats [7–9,14–17]. This observation is consistent with the consideration that the alteration of hepatic fatty acid metabolism is a crucial factor accounting for the serum lipid-lowering effect of n-3 fatty acid [4,7–9,14–17]. The dietary level of n-3 fatty acid ethyl ester (1%) used by Adan et al. [24] was lower than the level used in the current study (4%). In addition, they used DHA ethyl ester in the diet, but we added both DHA and EPA ethyl esters in our experimental diet. Therefore, it is possible that the differences in the dietary levels and compositions of n-3 fatty acid ethyl esters are responsible for the inconsistency between the results of Adan et al. [24] and the current study. In this context, Willumsen et al. [15–17] showed that EPA ethyl ester increased hepatic fatty acid oxidation and reduced serum lipid levels more than DHA ethyl ester in rats. In our study, dietary fish oil at a level (9.4%) much lower than that (20%) used by Asset et al. [2] profoundly lowered serum lipid levels in apoE-deficient mice. However, there is a possibility that differences in the n-3 fatty acid content and composition of this fish oil used in the present study and

their study can account for the discrepancy in results. They used fish oil purchased from Sigma-Aldrich Company (menhaden oil). Although the fatty acid composition of this fish oil was not provided in their study, a statement by the maker (http://www.sigmaaldrich.com/sigma/productinformationsheet/f8020pis.pdf) indicated that this product contains approximately 25% n-3 fatty acids as EPA and DHA with similar proportions. Therefore, the n-3 fatty acid content is considered to be higher (approximately 5%) in their fish oil diet than in the fish oil diet used in the present study (4%, Table 1). Moreover, the dietary level of EPA that is more active than DHA in reducing serum lipids levels [15–17] is considered to be higher in the fish oil diet used by Asset et al. [2] than in our fish oil diet. Given the above, it is unlikely that the differences in the n-3 fatty acid content and composition of experimental diets can account for the inconsistency of the results. Therefore, the present study indicates that a lack of apoE expression does not necessarily accompany deficits in the n-3 fatty acid–dependent regulation of serum lipid levels.

Our study raises the possibility that apoE-knockout (*Apoe*^{tm1Unc}) mice generated by gene targeting do not represent a suitable animal model for examining the physiological activity of dietary factors affecting lipid metabolism. It should be stated that the genetic background is different between the *Apoe*^{shl} mice (BALB/c) used in the current study and *Apoe*^{tm1Unc} mice (C57BL/6J) used by others [2,24]. It has been demonstrated that fish oil effectively decreases serum lipid levels in the wild-type mice of various inbred strains [1–6]. Pennacchiotti et al. [1] compared serum lipid profiles between BALB/c mice fed diets containing either 9.2% fish oil or sunflower oil, and showed that the former compared to the latter caused about a 50% decrease in serum triacylglycerol and cholesterol concentrations. Other studies [2–4] showed that fish oil, compared to saturated fats or fats rich in linoleic acid, caused 30–45% and 25–55% decreases in serum triacylglycerol and cholesterol levels, respectively, in C57BL/6J mice. Similar reductions in serum triacylglycerol and cholesterol levels were confirmed in NZB/W F1 mice [5] and SV 129 mice [6]. However, it is possible that the impact of fish oil on serum lipid levels depends on genetic background in apoE-deficient mice. Also, it is possible that the *Apoe*^{shl} mice used in the present study have a mutation not only in the apoE gene but also in some other gene or genes involved in the regulation of lipid metabolism and therefore have different responses to dietary n-3 fatty acids. Studies to compare the physiological activity of n-3 fatty acids in affecting serum lipid levels among *Apoe*^{tm1Unc} and *Apoe*^{shl} mice of different genetic backgrounds are necessary to address these considerations.

There is a general consensus that the physiological activity of fish oil in increasing hepatic fatty acid oxidation is ascribable to EPA and DHA. These fatty acids may increase hepatic fatty acid oxidation through the activation of peroxisome proliferator activated receptor (PPARα) [12,43,44], a member of the nuclear receptor superfamily. In fact, several studies [14–17] have demonstrated that highly purified EPA and DHA in the form of ethyl esters increase hepatic β-oxidation, and the activity and gene expression of hepatic fatty acid oxidation enzymes in rats. Although these observations support the notion that EPA and DHA are the components accounting for the physiological activity of fish oil in increasing hepatic fatty acid oxidation, a study to compare the physiological activity of EPA and DHA ethyl esters and fish oil in affecting hepatic fatty acid oxidation has been lacking. In the current study, the diet containing n-3 fatty acids as ethyl esters, compared to the fish oil diet containing equivalent amounts of n-3 fatty acids in the form of triacylglycerol, was less effective in increasing activity and mRNA levels of hepatic fatty acid oxidation enzymes in apoE-deficient mice. Moreover, fish oil compared to ethyl esters was more effective in increasing the gene expression of cytochrome P-450 IV A1 involved in the ω-oxidation of fatty acids and known to be induced through a PPARα-dependent mechanism [41]. However, fish oil and ethyl esters were equally effective in decreasing the activity and mRNA levels of lipogenic enzymes and mRNA level of sterol regulatory element binding protein-1 involved in regulating the gene expression of lipogenic enzymes [4]. Therefore, the difference in the hepatic fatty acid oxidation rate may account for the different effects of fish oil and ethyl esters on serum lipid levels in apoE-deficient mice. Some studies in rats [45] and humans [46] indicated that EPA and DHA are less absorbable in the small intestine in the form of ethyl ester than in the form of triacylglycerol, but controversy exists [47,48]. The present observations on hepatic n-3 fatty acid content favor the notion that EPA and DHA are less absorbable when given as ethyl esters. The difference in the amount of n-3 fatty acids available in liver between apoE-deficient mice fed ethyl esters and fish oil may account for the different effects of these dietary lipids on hepatic fatty acid oxidation.

In conclusion, in contrast to previous observations made in apoE-knockout (*Apoe*^{tm1Unc}) mice [2,24], n-3 fatty acids in the form of either ethyl esters or fish oil greatly decreased serum lipid levels accompanying parallel changes in the activity and mRNA levels of enzymes involved in hepatic fatty acid oxidation and synthesis in an alternative apoE-deficient murine model, BALB/c.KOR-*Apoe*^{shl} mice. Therefore, it is considered that a lack of apoE expression does not necessarily accompany deficits in the n-3 fatty acid–dependent regulation of serum lipid levels and hepatic fatty acid metabolism. Compared to apoE-knockout mice, our apoE-deficient mice may serve as a superior animal model of hyperlipidemia with which to clarify the physiological activity of nutritional factors affecting lipid metabolism.

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