

# Polyunsaturated Fatty Acids Ameliorate Hepatic Steatosis in Obese Mice by SREBP-1 Suppression

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**Leptin-deficient *ob/ob* mice show many characteristics of obesity, including excess peripheral adiposity as well as severe hepatic steatosis, at least in part, due to increased hepatic lipogenesis. Polyunsaturated fatty acids (PUFAs) are not only ligands for peroxisome proliferator-activated receptor (PPAR)  $\alpha$  but are also negative regulators of hepatic lipogenesis, which is thought to be mediated by the repression of sterol regulatory element-binding protein (SREBP)-1. We have previously shown that the disruption of SREBP-1 in *ob/ob* mice decreased their liver triglyceride storage. To examine whether PUFAs could reduce hepatic triglyceride deposition, we challenged *ob/ob* mice with dietary PUFA. It is demonstrated that PUFA markedly decreased the mature form of SREBP-1 protein and thereby reduced the expression of lipogenic genes such as fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) in the livers of *ob/ob* mice. Consequently, the liver triglyceride content and plasma alanine aminotransferase (ALT) levels were decreased. Furthermore, both hyperglycemia and hyperinsulinemia in *ob/ob* mice were improved by PUFA administration, similar to the effect of PPAR $\alpha$  activators. In conclusion, PUFAs ameliorate obesity-associated symptoms, such as hepatic steatosis and insulin resistance, presumably through both down-regulation of SREBP-1 and activation of PPAR $\alpha$ . (HEPATOLOGY 2003;38:1529-1539.)**

Sterol regulatory element-binding proteins (SREBPs) are members of the basic helix-loop-helix leucine zipper family of transcription factors that regulate fatty acid and cholesterol synthesis (reviewed in Brown and Goldstein<sup>1</sup>). Unlike other members of the family, SREBPs are synthesized as precursors bound to the endoplasmic reticulum and nuclear envelope and are released from the membrane into the nucleus as mature proteins by cleavage processes. To date, 3 isoforms of SREBP, -1a, -1c, and -2, have been identified and characterized. The predominant SREBP-1 isoform in liver and adipose tissue is SREBP-1c. Whereas SREBP-2 plays a crucial role in regulation of cholesterol synthesis, SREBP-1c controls

the transcription and expression of lipogenic enzymes such as fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) (reviewed in Shimano<sup>2</sup> and Horton et al.<sup>3</sup>). It is remarkable that SREBP-1c regulates not only the synthetic rate of triglycerides but also the amount of their storage in the liver.<sup>4,5</sup> Thus, SREBP-1 has been revealed to be a promising target for hepatic steatosis (fatty livers) from a therapeutic point of view.

The leptin-deficient *ob/ob* mouse model of obesity exhibits severe obesity and obesity-related symptoms, including hepatic steatosis and insulin resistance (reviewed in Bray and York<sup>6</sup>). The livers of *ob/ob* mice have an increase in triglyceride content, probably because of the increased lipogenesis paralleled by elevated messenger RNA (mRNA) expression and enzymatic activity of several lipogenic enzymes such as FAS and SCD1.<sup>6,7</sup> Recently, it has been reported that both SREBP-1c mRNA and its active nuclear protein are increased in *ob/ob* mouse livers.<sup>8</sup> Furthermore, we have demonstrated in a previous report<sup>5</sup> that the disruption of the SREBP-1 gene in *ob/ob* mice leads to marked amelioration of hepatic steatosis.

Dietary polyunsaturated fatty acids (PUFAs) of the n-6 and n-3 families are well established as negative regulators of hepatic lipogenesis (reviewed in Clark and Jump<sup>9</sup>). Recently, others and we have shown that the suppressive

*Abbreviations:* SREBP, sterol regulatory element-binding protein; FAS, fatty acid synthase; SCD, stearoyl-CoA desaturase; PUFA, polyunsaturated fatty acid(s); ACL, ATP citrate lyase; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PPAR, peroxisome proliferator-activated receptor.

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effects of PUFA on lipogenic enzymes are mediated by their reduction of mature SREBP-1 protein in the liver.<sup>10-13</sup> The primary mechanism by which PUFAs suppress SREBP-1 is presumably by cleaving the precursor protein as well as by the reduction of both transcription rate and mRNA stability.<sup>14,15</sup> Interestingly, PUFAs selectively decrease SREBP-1, not affecting SREBP-2.

Considering these findings, we hypothesized that PUFA would be effective for the treatment of hepatic steatosis. To test this, we examined the effects of PUFA on hepatic steatosis in the *ob/ob* mouse. We show that, in *ob/ob* mice, PUFAs ameliorate hepatic steatosis through a reduction of mature SREBP-1 protein. In addition, the administration of PUFA resulted in marked improvement of insulin resistance.

## Materials and Methods

**Materials.** Triolein (99% grade) and fenofibrate were purchased from Wako Pure Chemicals (Tokyo, Japan) and Sigma (St. Louis, MO), respectively. Eicosapentaenoic acid (EPA) ethyl ester (95% grade) was provided from Mochida pharmaceutical (Tokyo, Japan) and pioglitazone (100.0% pure) from Takeda pharmaceutical (Tokyo, Japan). Fish oils (extracted from tuna; containing 24.8% docosahexaenoic acid [DHA] and 8.1% EPA) were provided from NOF (Tokyo, Japan). Standard laboratory chow (composed of 60% carbohydrate, 13% fat, and 27% protein on a caloric basis; Oriental MF, Oriental Yeast, Tokyo, Japan), high-carbohydrate, fat-free diet (70% sucrose; 10% starch; and 20% casein supplemented with methionine, vitamins, and minerals), and high-protein, fat-free diet (100% casein and no carbohydrate supplemented with methionine, vitamins, and minerals) were obtained from Oriental Yeast (Tokyo, Japan).

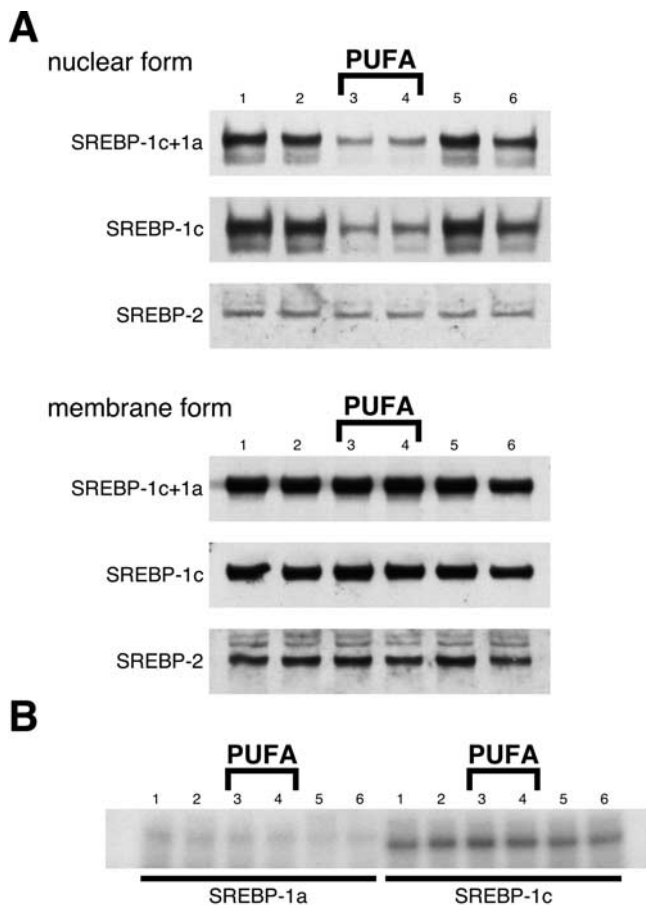
**Animals.** Heterozygous *Lep<sup>ob/+</sup>* C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Eight-week-old wild-type and *ob/ob* male littermates were used in all experiments. Genotypes at the leptin locus were determined by a PCR-based, restricted fragment length polymorphism analysis as described previously.<sup>5</sup> Mice were housed in a temperature-controlled environment with a 12-hour light/dark cycle (light: 10:00 AM to 10:00 PM) and given free access to water and a standard chow diet for the 1-week adaptation period. Each group of 4 animals (kept all together in 1 cage) was fed a control high-carbohydrate, fat-free chow or that supplemented with either 15% triolein, 15% triolein plus 5% EPA ethyl ester (approximately 5 g/kg/d), 20% fish oil, 0.125% fenofibrate, or 0.05% pioglitazone for 7 days. We determined this duration based on the preliminary data that 7-day administration of fish oil decreased the serum ala-

nine aminotransferase (ALT) level to the same extent as was observed at the 14th day, whereas 3-day feeding was not sufficient. After collecting blood samples from the retroorbital sinus, animals were killed by cervical dislocation in an early phase of the light cycle in a nonfasted state. SREBP-1c transgenic mice over-expressing amino acids 1–436 of human SREBP-1c under control of the rat PEPCK promoter (TgSREBP1) were made as previously described.<sup>4</sup> A line homozygous for the transgene was established and used. The diets used for TgSREBP1 mice experiments were based on the high-protein, fat-free diet to maximize the transgene expression and mixed with 15% triolein, 15% triolein plus 5% EPA ethyl ester, or 20% fish oil. All experiments were repeated at least twice, and the data from a representative experiment were shown.

**Blood Chemistries and Liver Lipid Analyses.** Enzymatic assay kits were used for the determination of cholesterol (Determiner TC: cholesterol oxidase method; Kyowa Medex, Tokyo, Japan), triglycerides (TG LH: lipoprotein lipase method; Wako Pure Chemicals, Tokyo, Japan), nonesterified fatty acids (NEFA C: acyl-CoA oxidase method; Wako Pure Chemicals) and ALT (transaminase C-II: POP TOOS method; Wako Pure Chemicals). Plasma glucose was measured by ANTSENSE II (Bayer Medical, Tokyo, Japan) based on immobilized glucose oxidase membrane/hydrogen peroxide electrode method. Plasma insulin was determined by the mouse insulin ELISA kit (Wako Pure Chemicals). The content of cholesterol and triglycerides in liver was measured as described previously.<sup>16</sup>

**RNA Isolation and Northern Blotting.** Total RNA from liver and epididymal fat pad was isolated with Trizol Reagent (Invitrogen), and 10  $\mu$ g RNA samples were equally pooled from 4 mice of each group and (2.5  $\mu$ g RNA for each mouse) were run on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane. The cDNA probes used were cloned as previously described.<sup>17</sup> The probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using Megaprime DNA Labelling System kit (Amersham Biosciences). The membranes were hybridized with the radiolabeled probe in Rapid-hyb Buffer (Amersham Biosciences) at 65°C. The membranes were washed 3 times (10 minutes each) in 0.1X SSC, 0.1% SDS at 65°C. Blots were exposed to Kodak XAR-5 film and the BAS imaging plate for BAS2000 BIO IMAGING ANALYZER (Fuji Photo Film, Tokyo, Japan). The quantification results obtained with BAS2000 system were normalized to the signal generated from 36B4 (acidic ribosomal phosphoprotein P0) mRNA.

**RNase Protection Assay.** RNase protection assay was performed as described previously.<sup>18</sup> Briefly, after linear-

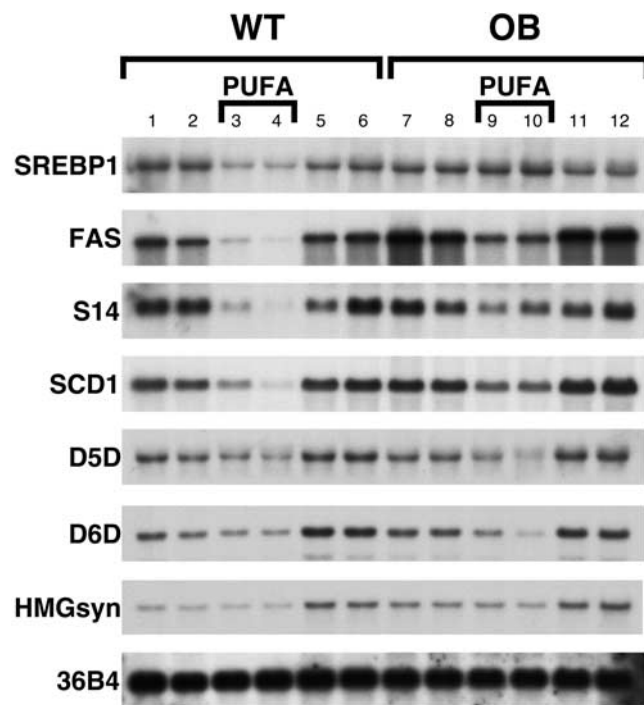


**Fig. 1.** PUFAs reduce mature SREBP-1 in the nuclei from *ob/ob* mice livers. (A) Immunoblot analysis of SREBP-1 and -2 in nuclear extracts and membrane fractions from livers of *ob/ob* mice are shown. Mice (4 male *ob/ob* mice for each group) were fed a high-carbohydrate fat-free diet (**lane 1**) or a high-carbohydrate diet supplemented with 15% triolein (**lane 2**), 5% EPA ethyl ester plus 15% triolein (**lane 3**), 20% fish oil (**lane 4**), 0.125% fenofibrate (**lane 5**), or 0.05% pioglitazone (**lane 6**) for 7 days before being killed in a nonfasted state. Aliquots of nuclear extracts (20  $\mu$ g protein) and membrane proteins (30  $\mu$ g) from pooled livers of each group were subjected to immunoblot analysis. The primary antibodies used were polyclonal anti-mouse SREBP-1 (1c plus 1a and 1c-specific) and -2. (B) Quantification of SREBP-1a and -1c by RNase protection assay are shown. **Lanes** are the same as above. The protected fragments for SREBP-1a and -1c contain the same number of cytidine phosphates (32 cytidines), so the intensity of each band was proportional to the amount of each isoform in the samples. Series of experiments were performed twice for the same number of animals, and the data from a representative experiment are shown.

ization of the template plasmid DNA with Hind III, antisense RNA probes for SREBP-1a and -1c were transcribed with [ $\alpha$ - $^{32}$ P]CTP using bacteriophage T7 RNA polymerase (MAXIscript *in vitro* transcription kit; Ambion) and purified by electrophoresis on 5% polyacrylamide gel. Then, aliquots of probes and total RNA (10  $\mu$ g) pooled within each group (2.5  $\mu$ g RNA for each mouse) were mixed and subjected to the RNase protection assay using HybSpeed RPA kit (Ambion). After digestion with RNase A/T1, protected fragments were

separated on 8 mol/L urea/10% polyacrylamide gels. The protected fragments for SREBP-1a and -1c contained the same number of cytidine phosphates (32 cytidines), so the intensity of each band was proportional to the amount of each isoform in the samples. The gels were subjected to Kodak XAR-5 film and the BAS2000 BIO IMAGING ANALYZER (Fuji Photo Film, Tokyo, Japan).

**Immunoblotting of SREBP Proteins.** Nuclear extracts and membrane fractions from mice livers were prepared as previously described.<sup>19</sup> Aliquots of nuclear (20  $\mu$ g) and membrane (30  $\mu$ g) proteins were subjected to SDS/PAGE. Immunoblot analysis was performed using the ECL Western Blotting Detection System kit (Amersham Biosciences) and exposed to Kodak XAR-5 film. The primary antibodies (rabbit polyclonal; No. 931 for mouse SREBP-1a and -1c, No. 772 for SREBP-1c, which



**Fig. 2.** PUFAs repress lipogenic enzymes in liver. Northern blot analysis from mice livers. Wild-type (**lanes 1-6**) and *ob/ob* (**lanes 7-12**) mice (4 mice for each group) were fed the indicated diet for 7 days and killed in a nonfasted state. Diets were as follows: **lanes 1** and **7**, a high-carbohydrate, fat-free diet; **lanes 2** and **8**, a high-carbohydrate diet with 15% triolein (C18:1 n-9); **lanes 3** and **9**, 5% EPA ethyl ester (C20:5 n-3) plus 15% triolein (C18:1 n-9); **lanes 4** and **10**, 20% tuna fish oil; **lanes 5** and **11**, 0.125% fenofibrate, a PPAR $\alpha$  agonist; and **lanes 6** and **12**, 0.05% pioglitazone, a PPAR $\gamma$  agonist. Total RNA (10  $\mu$ g) pooled equally from 4 mice was subjected to Northern blotting, followed by hybridization with the indicated cDNA probes. SREBP1, sterol regulatory element-binding protein-1; FAS, fatty acid synthase; S14, Spot 14; SCD1, stearoyl-CoA desaturase 1; D5D,  $\Delta$ 5-desaturase; D6D,  $\Delta$ 6-desaturase; HMGsyn, hydroxymethylglutaryl-CoA synthase. A cDNA probe for 36B4 (acidic ribosomal phosphoprotein P0) was used to confirm equal loading. Series of experiments were performed twice for the same number of animals, and the data from a representative experiment are shown.



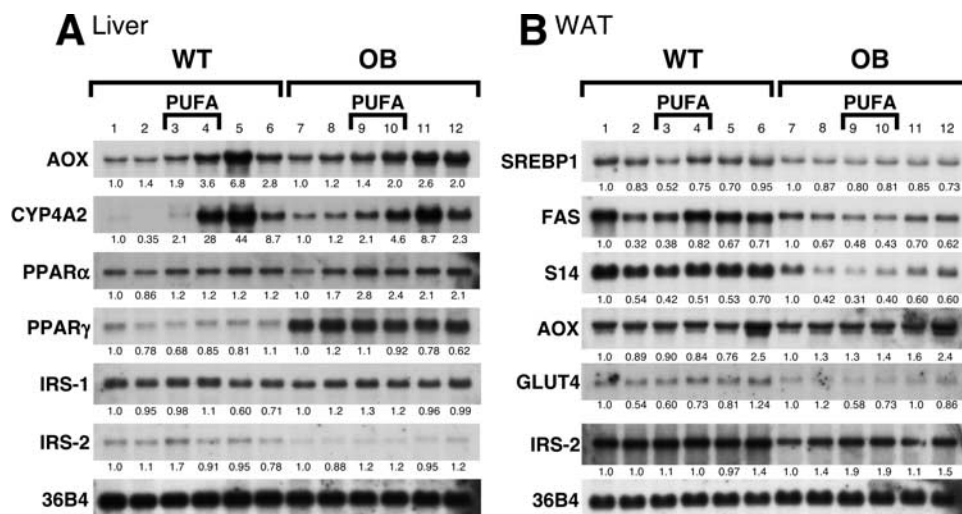


Fig. 3. PUFAs induce PPAR $\alpha$ -target genes. (A) Northern blot analysis from mice livers and (B) adipose tissue. Wild-type (lanes 1-6) and *ob/ob* (lanes 7-12) mice (4 mice for each group) were fed the indicated diet for 7 days and killed in a nonfasted state. Diets were as follows: lanes 1 and 7, a high-carbohydrate, fat-free diet; lanes 2 and 8, a high-carbohydrate diet with 15% triolein (C18:1 n-9); lanes 3 and 9, 5% EPA ethyl ester (C20:5 n-3) plus 15% triolein (C18:1 n-9); lanes 4 and 10, 20% tuna fish oil; lanes 5 and 11, 0.125% fenofibrate, a PPAR $\alpha$  agonist; and lanes 6 and 12, 0.05% pioglitazone, a PPAR $\gamma$  agonist. Total RNA (10  $\mu$ g), pooled equally from four mice, was subjected to Northern blotting, followed by hybridization with the indicated cDNA probes. AOX, acyl-CoA oxidase; CYP4A2, cytochrome P450 4A2; PPAR, peroxisome proliferator-activated receptor; IRS, insulin receptor substrate; SREBP1, sterol regulatory element-binding protein-1; FAS, fatty acid synthase; S14, Spot 14; GLUT4, glucose transporter 4. A cDNA probe for 36B4 (acidic ribosomal phosphoprotein P0) was used to confirm equal loading. The quantification results were obtained with BAS2000 system and normalized to the signal generated from 36B4 (acidic ribosomal phosphoprotein P0) mRNA, and the fold-changes against control were displayed below each blot. Series of experiments were performed twice for the same number of animals, and the data from a representative experiment are shown.

does not bind to SREBP-1a, and No. 528 for SREBP-2) were as previously described.<sup>17,20</sup>

**Fatty Acid Composition of Liver.** An aliquot (0.1 g) of liver samples snap-frozen by liquid nitrogen was homogenized in 1 mL of normal saline. The fatty acid composition was measured by gas chromatography at Bio-Medical Laboratories (Tokyo, Japan).<sup>21</sup> Briefly, total lipids in liver homogenates were extracted according to the Folch's procedure,<sup>22</sup> followed by transesterification of fatty acids with boron trifluoride-methanol at 100°C for 90 minutes. The methylated fatty acids were then extracted with hexane and analyzed using GC-17A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) and BPX70 capillary column (0.25 mm ID  $\times$  30 m, SGE International Ltd., Melbourne, Australia).

**Statistical Analysis.** Data are presented as means  $\pm$  SE. All data were analyzed by 2-tailed Student's *t* test against control. Differences were considered statistically significant at  $P < .05$ .

## Results

**Effects of PUFA on the Mature Form of SREBP-1 in *ob/ob* Mice Livers.** In wild-type mice, PUFAs have been shown to decrease the mature form of SREBP-1 in the liver.<sup>10-13</sup> To examine effects of PUFA on SREBP-1 in

leptin-deficient *ob/ob* mice livers in which SREBP-1 is dysregulated, they were fed a high-carbohydrate diet supplemented with PUFA (EPA or tuna fish oil enriched in DHA) or control diet for 7 days. As expected, immunoblot analysis of liver nuclear extracts showed that *ob/ob* mice fed a diet with EPA or fish oil for 7 days had  $\sim$ 3-fold lower SREBP-1 mature protein than those fed the control diet (high-carbohydrate without fat) (Fig. 1A). In contrast, monounsaturated fatty acids (oleate) did not reduce mature SREBP-1.

To clarify the mechanism by which PUFAs down-regulate mature SREBP-1 protein, the amounts of SREBP-1 (both precursor and mature forms in the membrane and nuclear extracts, respectively; Fig. 1A) as well as SREBP-1 mRNA levels (Fig. 1B) were compared between control and PUFA (EPA or fish oil)-fed *ob/ob* mice. In contrast to the mature protein, which was profoundly decreased by PUFA feeding, no reduction was observed in either mRNA or membrane-bound precursor protein levels. This indicates that PUFAs regulate the abundance of mature SREBP-1 protein mainly at a posttranslational level, presumably through cleavage processes.

RNase protection assay with probes that can discriminate SREBP-1a and -1c (Fig. 1B) also demonstrated that the isoform pattern of SREBP-1 is SREBP-1c-dominant

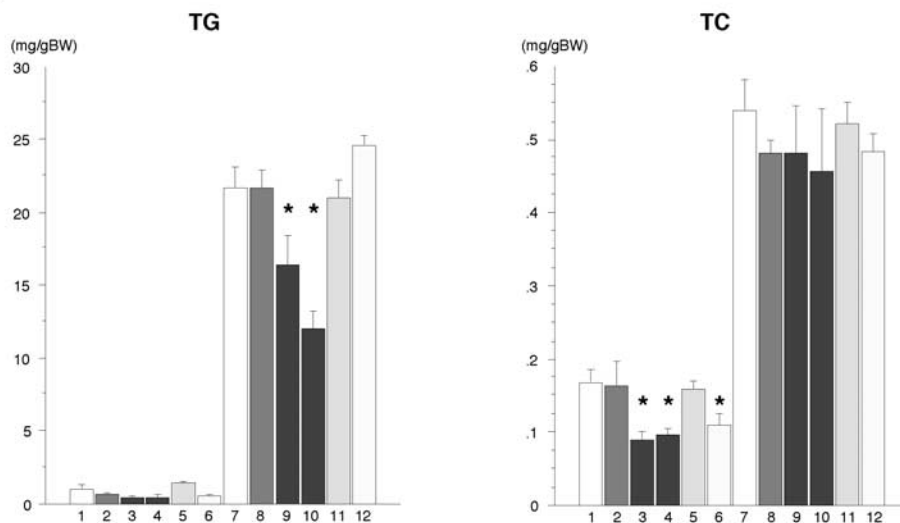
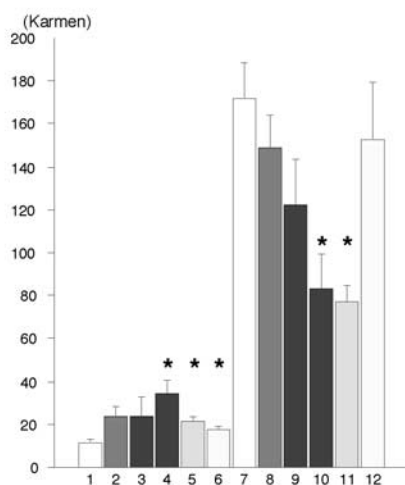
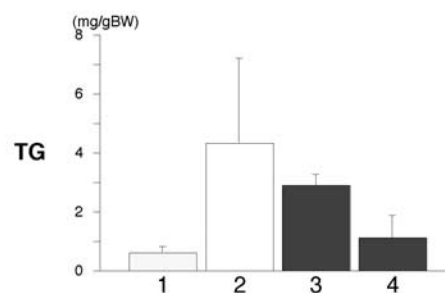
**A** Liver lipid / BW

Fig. 4. PUFAs ameliorate hepatic steatosis of *ob/ob* mice. (A) Liver triglyceride (TG) and total cholesterol (TC) content per body weight in wild-type (lanes 1-6) and *ob/ob* mice (lanes 7-12). Diets are as follows: lanes 1 and 7, a high-carbohydrate, fat-free diet; lanes 2 and 8, 15% triolein (C18:1 n-9); lanes 3 and 9, 5% EPA ethyl ester (C20:5 n-3) plus 15% triolein (C18:1 n-9); lanes 4 and 10, 20% tuna fish oil; lanes 5 and 11, 0.125% fenofibrate, a PPAR $\alpha$  agonist; and lanes 6 and 12, 0.05% pioglitazone, a PPAR $\gamma$  agonist. Each group consisted of 4 male mice except for lanes 7 and 9 ( $n = 8$ ). (B) Plasma ALT levels. Lanes are the same as above. (C) Liver lipid content of wild-type and SREBP-1 transgenic mice. Lanes are as follows: lane 1, wild-type mice fed with a high-carbohydrate, fat-free diet; lane 2, SREBP-1 transgenic (TgSREBP1) mice fed with a high-protein, fat-free diet; lane 3, TgSREBP1 mice fed with 5% EPA ethyl ester plus 15% triolein; lane 4, TgSREBP1 mice fed with 20% fish oil. Bars = SE. \*Denotes significance vs. control (lane 1 or 7) at  $P < .05$  (Student's  $t$  test). Series of experiments were performed 3 times for the same number of animals, and the data from a representative experiment are shown.

**B** Plasma ALT**C** TgSREBP1 liver lipid / BW

in *ob/ob* mice livers, which is the same as in wild-type mice.<sup>18</sup> This was further supported by Western blot analysis with an SREBP-1c-specific antibody (Fig. 1A).

Fenofibrate and pioglitazone, peroxisome proliferator-activated receptor (PPAR)  $\alpha$  and  $\gamma$  agonists, respectively, were also tested, but neither altered mature SREBP-1 levels. The amount of mature SREBP-2 protein did not change with any of these diets.

**Suppression of Lipogenic Gene Expressions by PUFA.** We compared the mRNA levels of genes encoding lipogenic enzymes in the livers of mice fed diets with or without PUFA (EPA or fish oil) for 7 days as measured by Northern blot analysis. Consistent with previous reports, dietary PUFA suppressed hepatic expression of SREBP-1-target lipogenic genes such as FAS and SCD1 in wild-type mice (Fig. 2). Similar suppression of these

genes was also observed in livers from *ob/ob* mice fed the PUFA diets. Meanwhile, suppression of these lipogenic genes was not observed in white adipose tissue from either group (Fig. 3).

The  $\Delta 5$ - and  $\Delta 6$ -desaturases, key players in the production of EPA and DHA, have been reported to be suppressed by PUFA.<sup>23,24</sup> We previously reported that they are dually regulated by SREBP-1 and PPAR $\alpha$  and suppressed by PUFA in wild-type mouse liver.<sup>25</sup> However, because PUFAs suppress SREBP-1 and at the same time activate PPAR $\alpha$ , it is unclear whether PUFAs decrease or increase these desaturases as an overall effect in various nutritional situations. In the current study, it was revealed that, in both wild-type and *ob/ob* mice, PUFA diets reduced the expression of these enzymes, whereas PPAR $\alpha$  activation by fenofibrate consistently increased their expression.

**Table 1. Representative Profile of Wild-Type and *ob/ob* Mice Used for the Experiment**

		1	2	3	4	5	6
<b>Wild-type</b>							
Body weight (g)	Pre	26.4 ± 1.9	26.4 ± 1.9	26.4 ± 1.6	26.5 ± 1.5	26.4 ± 1.3	26.4 ± 1.2
	At sacrifice	26.3 ± 2.1	26.8 ± 1.8	27.0 ± 1.5	25.1 ± 0.7	25.5 ± 1.1	26.8 ± 1.0
Liver weight (g)		1.35 ± 0.17	1.43 ± 0.13	1.25 ± 0.09	1.40 ± 0.10	2.33 ± 0.10*	1.53 ± 0.05
WAT weight (g)		0.28 ± 0.08	0.33 ± 0.05	0.53 ± 0.16	0.28 ± 0.05	0.20 ± 0.00	0.40 ± 0.07
Plasma data	TC (mg/dL)	72.5 ± 16.0	121.9 ± 5.5*	61.2 ± 9.3	57.2 ± 8.3	109.1 ± 7.4	106.0 ± 5.9
	TG (mg/dL)	64.2 ± 10.2	88.5 ± 11.1	55.1 ± 2.8	46.9 ± 2.8	66.7 ± 3.8	46.4 ± 2.2
<b><i>ob/ob</i></b>							
Body weight (g)	Pre	50.5 ± 2.5	50.3 ± 2.4	50.6 ± 2.1	50.4 ± 2.0	50.1 ± 1.9	50.4 ± 1.6
	At sacrifice	52.3 ± 2.1	53.6 ± 2.5	51.8 ± 2.4	51.8 ± 2.0	51.3 ± 1.7	54.1 ± 1.8
Liver weight (g)		6.12 ± 0.31	5.50 ± 0.20	4.58 ± 0.40*	4.60 ± 0.31*	5.35 ± 0.15	5.54 ± 0.32
WAT weight (g)		3.20 ± 0.31	3.30 ± 0.11	3.25 ± 0.41	3.62 ± 0.13	3.48 ± 0.15	3.63 ± 0.16
Plasma data	TC (mg/dL)	265.1 ± 28.0	284.5 ± 10.3	229.6 ± 8.0	330.6 ± 24.4	267.1 ± 13.4	191.5 ± 18.5
	TG (mg/dL)	115.9 ± 11.8	145.6 ± 26.8	79.6 ± 0.6*	147.0 ± 25.7	79.6 ± 4.0*	69.2 ± 10.0*

NOTE. Diets are as follows: 1, a high-carbohydrate fat-free diet; 2, a high-carbohydrate diet with 15% triolein (C18:1 n-9); 3, 5% EPA ethyl ester (C20:5 n-3) plus 15% triolein (C18:1 n-9); 4, 20% tuna fish oil; 5, 0.125% fenofibrate, a PPAR $\alpha$  agonist; 6, 0.05% pioglitazone, a PPAR $\gamma$  agonist. Each value is mean  $\pm$  SE.

Abbreviations: TC, total cholesterol; TG, triglycerides.

\*Significance vs. control at  $P < .05$  by Student's  $t$  test.

In contrast, these lipids did not affect genes involved in cholesterol synthesis, such as hydroxymethylglutaryl (HMG)-CoA synthase, that are mainly regulated by SREBP-2 (Fig. 3). These results are consistent with the data shown in Fig. 1 in which PUFA did not affect the amount of mature SREBP-2.

**Effects on PPAR $\alpha$ -Regulated Genes.** It is well known that PUFAs are ligands for PPAR $\alpha$ ,<sup>26,27</sup> and some biologic effects of PUFA such as stimulation of peroxisomal and microsomal fatty acid oxidation are mediated by PPAR $\alpha$ .<sup>28</sup> Therefore, we assessed PUFA effects on the hepatic PPAR $\alpha$  pathway. As expected, mRNA levels of PPAR $\alpha$ -target genes such as PPAR $\alpha$  itself, acyl-CoA oxidase (AOX), and cytochrome P-450 4A2 (CYP4A2) were induced by PUFA (Fig. 3). In particular, fish oil (tuna, rich in DHA) had more potent effects than EPA on PPAR $\alpha$ -mediated pathways in both wild-type and *ob/ob* mice.

**Effects of PUFA on Liver Lipid Content.** To determine whether the effects of PUFA reduced hepatic steatosis in *ob/ob* mice, liver triglyceride content was measured (Fig. 4). In both wild-type and *ob/ob* mice, PUFA (EPA and fish oil) significantly ( $P < .05$ ) decreased hepatic triglyceride content per body weight. During the feeding period, mean body weights were not different among each group (Table 1), suggesting that the decrease in hepatic triglycerides was not due to reduced food intake.

**Reduction of ALT by PUFA.** ALT is a well-known marker for evaluation of liver injury and is elevated in animals with hepatic steatosis, including *ob/ob* mice. We determined plasma ALT levels by enzymatic assay. Although there were no differences among each dietary group in wild-type mice, PUFA (EPA or fish oil) lowered the elevated ALT levels in *ob/ob* mice (Fig. 4). These data

are consistent with our finding that PUFA ameliorated hepatic steatosis.

**Effects of PUFA on Triglyceride Content in Tg-SREBP1 Mice Livers.** To investigate whether the reduction of triglyceride in liver can be ascribed to the decrease in mature SREBP-1, we examined a transgenic mouse model, which forcibly expresses a mature form of SREBP-1 protein in the liver (TgSREBP1). Consistent with a previous report,<sup>10</sup> dietary PUFA did not decrease the mature SREBP-1 protein or the mRNA levels of lipogenic genes in the liver of TgSREBP1 mice (data not shown). Accordingly, the triglyceride content of EPA-fed mice was almost equal to mice fed the control diet (Fig. 4). Intriguingly, the fish oil administration still caused a remarkable reduction in the triglyceride content elevated by the over-expressed SREBP-1. These data suggest that fish oil has some additional effects beyond those mediated by SREBP-1.

**Effects on Fatty Acid Composition in Liver.** We measured the composition of fatty acids in liver total lipids by gas chromatography (Table 2, Fig. 5). The fatty acids that can be synthesized *de novo* by lipogenic enzymes such as palmitate (C16:0), palmitoleate (C16:1 n-7), and oleate (C18:1 n-9) were reduced by PUFA. Because these fatty acids dominate liver lipids, this resulted in an overall reduction in the total content of fatty acids in PUFA-fed mice. In the animals fed fish oil, EPA (C20:5 n-3) and DHA (C22:6 n-3) were abundantly distributed in the liver in both wild-type and *ob/ob* mice. However, mice given EPA showed similarly low levels of DHA compared with control animals, despite the availability of EPA for metabolism to DHA by desaturation and elongation.

**PUFA Effects on Insulin Resistance.** To evaluate insulin sensitivity, we also examined some markers of glu-

Table 2. Fatty Acid Composition in the Liver

	1	2	3	4	5	6
Wild-type ( $\mu\text{g/g}$ body weight)						
C12:0	0.22 $\pm$ 0.22	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.34 $\pm$ 0.34	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
C14:0	6.88 $\pm$ 0.64	4.93 $\pm$ 0.19*	2.94 $\pm$ 4.58*	3.54 $\pm$ 0.73*	9.40 $\pm$ 1.02	4.84 $\pm$ 0.22*
C14:1 n-5	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
C16:0	327.2 $\pm$ 24.0	342.6 $\pm$ 23.4	251.9 $\pm$ 15.5*	394.9 $\pm$ 13.3*	1084.0 $\pm$ 96.0*	431.8 $\pm$ 30.8*
C16:1 n-7	77.2 $\pm$ 12.8	51.7 $\pm$ 3.4	20.1 $\pm$ 2.8*	20.9 $\pm$ 3.3*	248.0 $\pm$ 30.7*	97.5 $\pm$ 8.3
C18:0	106.6 $\pm$ 13.4	125.1 $\pm$ 11.3	118.5 $\pm$ 5.8	167.1 $\pm$ 10.2*	165.6 $\pm$ 19.5*	118.8 $\pm$ 6.7
C18:1 n-9	475.5 $\pm$ 25.0	499.7 $\pm$ 19.2	286.6 $\pm$ 43.9*	175.4 $\pm$ 52.6*	975.8 $\pm$ 106.9*	481.9 $\pm$ 43.3
C18:2 n-6	114.5 $\pm$ 32.2	113.0 $\pm$ 17.0	93.7 $\pm$ 16.1	156.8 $\pm$ 53.0	254.0 $\pm$ 19.7*	64.4 $\pm$ 4.4
C18:3 n-6	2.14 $\pm$ 1.12	1.62 $\pm$ 0.56	0.36 $\pm$ 0.36	1.33 $\pm$ 0.89	5.69 $\pm$ 0.73*	0.83 $\pm$ 0.48
C18:3 n-3	0.52 $\pm$ 0.52	0.44 $\pm$ 0.44	2.42 $\pm$ 0.45*	3.54 $\pm$ 1.16	0.59 $\pm$ 0.59	0.00 $\pm$ 0.00
C20:0	2.38 $\pm$ 0.25	3.00 $\pm$ 0.03*	2.75 $\pm$ 0.26	3.61 $\pm$ 0.13*	3.09 $\pm$ 0.31	2.00 $\pm$ 0.09
C20:1 n-9	19.23 $\pm$ 1.33	19.35 $\pm$ 0.59	8.01 $\pm$ 1.36*	7.10 $\pm$ 3.02*	20.14 $\pm$ 2.57	14.78 $\pm$ 1.70
C20:2 n-6	3.58 $\pm$ 1.06	3.14 $\pm$ 0.60	1.13 $\pm$ 0.40	2.13 $\pm$ 0.92	5.24 $\pm$ 0.40	1.46 $\pm$ 0.06
C20:3 n-9	24.18 $\pm$ 7.49	21.95 $\pm$ 2.35	0.95 $\pm$ 0.55*	1.26 $\pm$ 0.88*	27.58 $\pm$ 5.42	67.52 $\pm$ 7.16*
C20:3 n-6	19.38 $\pm$ 2.72	25.77 $\pm$ 3.04	3.94 $\pm$ 0.80*	5.67 $\pm$ 1.93*	90.98 $\pm$ 8.43*	19.58 $\pm$ 1.08
C20:4 n-6	85.6 $\pm$ 11.5	104.0 $\pm$ 11.4	23.9 $\pm$ 1.6*	107.4 $\pm$ 8.9	221.4 $\pm$ 25.3*	102.2 $\pm$ 5.9
C20:5 n-3	4.38 $\pm$ 0.7	5.07 $\pm$ 0.4	201.9 $\pm$ 16.6*	54.9 $\pm$ 8.1*	15.5 $\pm$ 0.8*	6.1 $\pm$ 0.4
C22:0	4.19 $\pm$ 0.95	4.68 $\pm$ 0.21	4.41 $\pm$ 0.44	7.06 $\pm$ 1.51	5.44 $\pm$ 0.58	4.32 $\pm$ 0.21
C22:1 n-9	1.03 $\pm$ 0.37	1.50 $\pm$ 0.08	1.32 $\pm$ 0.10	0.43 $\pm$ 0.43	0.00 $\pm$ 0.00*	0.38 $\pm$ 0.38
C22:4 n-6	3.86 $\pm$ 1.44	2.41 $\pm$ 0.30	0.00 $\pm$ 0.00*	1.90 $\pm$ 1.00	9.53 $\pm$ 1.13*	2.29 $\pm$ 0.19
C22:5 n-3	4.13 $\pm$ 0.94	3.68 $\pm$ 0.37	58.34 $\pm$ 5.65*	29.95 $\pm$ 9.42*	13.77 $\pm$ 0.51*	3.53 $\pm$ 0.20
C22:6 n-3	117.9 $\pm$ 19.4	100.9 $\pm$ 7.6	103.0 $\pm$ 15.4	460.4 $\pm$ 64.8*	169.5 $\pm$ 13.0	89.6 $\pm$ 5.1
C24:0	2.79 $\pm$ 0.76	2.86 $\pm$ 0.34	3.48 $\pm$ 0.36	3.33 $\pm$ 0.64	3.31 $\pm$ 0.34	2.95 $\pm$ 0.35
C24:1 n-9	5.47 $\pm$ 1.06	6.59 $\pm$ 0.70	5.00 $\pm$ 0.71	5.15 $\pm$ 0.84	8.73 $\pm$ 0.96	5.85 $\pm$ 0.30
<i>ob/ob</i> ( $\mu\text{g/g}$ body weight)						
C12:0	4.52 $\pm$ 0.35	3.99 $\pm$ 0.47*	1.67 $\pm$ 0.60	2.71 $\pm$ 0.95	4.02 $\pm$ 0.36	5.08 $\pm$ 0.23
C14:0	169.3 $\pm$ 7.2	159.3 $\pm$ 22.7	84.0 $\pm$ 15.3*	90.9 $\pm$ 16.1*	128.4 $\pm$ 4.2*	191.9 $\pm$ 9.0
C14:1 n-5	7.72 $\pm$ 0.70	5.75 $\pm$ 1.27	1.52 $\pm$ 0.98*	1.97 $\pm$ 0.69*	3.47 $\pm$ 0.12*	9.47 $\pm$ 0.84
C16:0	6397 $\pm$ 198	5797 $\pm$ 553	3328 $\pm$ 544*	3488 $\pm$ 554*	5932 $\pm$ 289	6553 $\pm$ 477
C16:1 n-7	1673 $\pm$ 153	1458 $\pm$ 231	753 $\pm$ 141*	912 $\pm$ 136*	1563 $\pm$ 60	2375 $\pm$ 155*
C18:0	587.6 $\pm$ 28.2	490.5 $\pm$ 13.3*	374.7 $\pm$ 36.4*	363.2 $\pm$ 62.0*	416.6 $\pm$ 18.1*	464.2 $\pm$ 29.1*
C18:1 n-9	12964 $\pm$ 214	13207 $\pm$ 1229	7661 $\pm$ 1563*	6551 $\pm$ 1137*	11014 $\pm$ 656*	15045 $\pm$ 639*
C18:2 n-6	1260.3 $\pm$ 242.7	1308.2 $\pm$ 22.3	921.5 $\pm$ 87.7	1033.6 $\pm$ 146.4	1113.5 $\pm$ 93.1	986.2 $\pm$ 106.9
C18:3 n-6	12.83 $\pm$ 2.18	11.67 $\pm$ 0.92	4.21 $\pm$ 0.81*	6.53 $\pm$ 1.01*	10.14 $\pm$ 1.02	9.77 $\pm$ 1.86
C18:3 n-3	45.6 $\pm$ 9.7	51.4 $\pm$ 2.8	53.0 $\pm$ 4.8	79.9 $\pm$ 17.3	35.1 $\pm$ 3.6	24.5 $\pm$ 2.2
C20:0	7.48 $\pm$ 0.56	6.34 $\pm$ 0.52	5.88 $\pm$ 0.91	8.51 $\pm$ 3.02	6.47 $\pm$ 0.73	13.21 $\pm$ 1.24
C20:1 n-9	386.1 $\pm$ 22.1	412.3 $\pm$ 60.0	177.9 $\pm$ 44.9*	148.1 $\pm$ 26.1*	286.9 $\pm$ 23.4*	535.6 $\pm$ 13.1*
C20:2 n-6	31.4 $\pm$ 6.2	37.6 $\pm$ 0.6	20.0 $\pm$ 3.0	19.9 $\pm$ 2.4	21.8 $\pm$ 2.9	24.4 $\pm$ 3.5
C20:3 n-9	37.61 $\pm$ 4.07	36.39 $\pm$ 2.85	13.71 $\pm$ 1.89*	10.19 $\pm$ 0.79*	26.86 $\pm$ 3.19	59.54 $\pm$ 8.41
C20:3 n-6	85.2 $\pm$ 10.8	91.2 $\pm$ 2.1	41.5 $\pm$ 5.2*	38.5 $\pm$ 3.7*	82.4 $\pm$ 5.7	70.8 $\pm$ 6.1
C20:4 n-6	134.6 $\pm$ 41.8	164.4 $\pm$ 2.6	76.2 $\pm$ 9.2	152.3 $\pm$ 18.1	140.1 $\pm$ 8.6	146.7 $\pm$ 14.2
C20:5 n-3	54.6 $\pm$ 6.0	57.9 $\pm$ 1.3	387.8 $\pm$ 13.8*	382.1 $\pm$ 70.6*	65.4 $\pm$ 2.1	45.7 $\pm$ 4.1
C22:0	4.61 $\pm$ 0.48	2.29 $\pm$ 0.80*	3.23 $\pm$ 0.23*	3.09 $\pm$ 1.15	1.50 $\pm$ 0.87*	2.44 $\pm$ 0.88
C22:1 n-9	9.7 $\pm$ 0.5	7.5 $\pm$ 0.5*	1.4 $\pm$ 0.8*	0.6 $\pm$ 0.6*	5.6 $\pm$ 0.7*	13.0 $\pm$ 0.5*
C22:4 n-6	11.3 $\pm$ 1.3	12.3 $\pm$ 0.7	3.8 $\pm$ 0.6*	11.0 $\pm$ 1.2	5.4 $\pm$ 1.1*	10.3 $\pm$ 1.7
C22:5 n-3	64.3 $\pm$ 9.4	74.2 $\pm$ 3.4	266.4 $\pm$ 28.4*	221.3 $\pm$ 33.6*	38.5 $\pm$ 3.0*	38.1 $\pm$ 3.7*
C22:6 n-3	350.2 $\pm$ 34.3	378.9 $\pm$ 8.6	317.4 $\pm$ 33.3	1130.7 $\pm$ 168.8*	244.7 $\pm$ 11.8*	185.9 $\pm$ 20.9*
C24:0	2.4 $\pm$ 0.8	1.4 $\pm$ 0.8	2.4 $\pm$ 0.8	2.2 $\pm$ 0.8	2.0 $\pm$ 0.7	0.6 $\pm$ 0.6
C24:1 n-9	12.0 $\pm$ 0.8	11.3 $\pm$ 0.5	9.6 $\pm$ 0.6	10.2 $\pm$ 1.1	9.9 $\pm$ 0.4	11.3 $\pm$ 0.7

NOTE. Diets are as follows: 1, a high-carbohydrate fat-free diet; 2, a high-carbohydrate diet with 15% triolein (C18:1 n-9); 3, 5% EPA ethyl ester (C20:5 n-3) plus 15% triolein (C18:1 n-9); 4, 20% tuna fish oil; 5, 0.125% fenofibrate, a PPAR $\alpha$  agonist; 6, 0.05% pioglitazone, a PPAR $\gamma$  agonist. Each value is mean  $\pm$  SE.

\*Significance vs. control at  $P < .05$  by Student's  $t$  test.

cose-lipid metabolism (Fig. 6). It has been suggested that PPAR $\alpha$  agonists reduce insulin resistance,<sup>29,30</sup> and, in this study, fenofibrate did ameliorate hyperinsulinemia and hyperglycemia in the *ob/ob* mice, although to a lesser degree than the established insulin sensitizer pioglitazone. Intriguingly, PUFA also reduced serum glucose and insu-

lin levels nearly to the same level as fenofibrate. By Northern blot analysis, the expression of insulin receptor substrate (IRS)-2, a key molecule in insulin signaling, in both liver and adipose tissue of *ob/ob* mice were shown to be lowered, but its expression was slightly restored by the PUFA administration (Fig. 3).



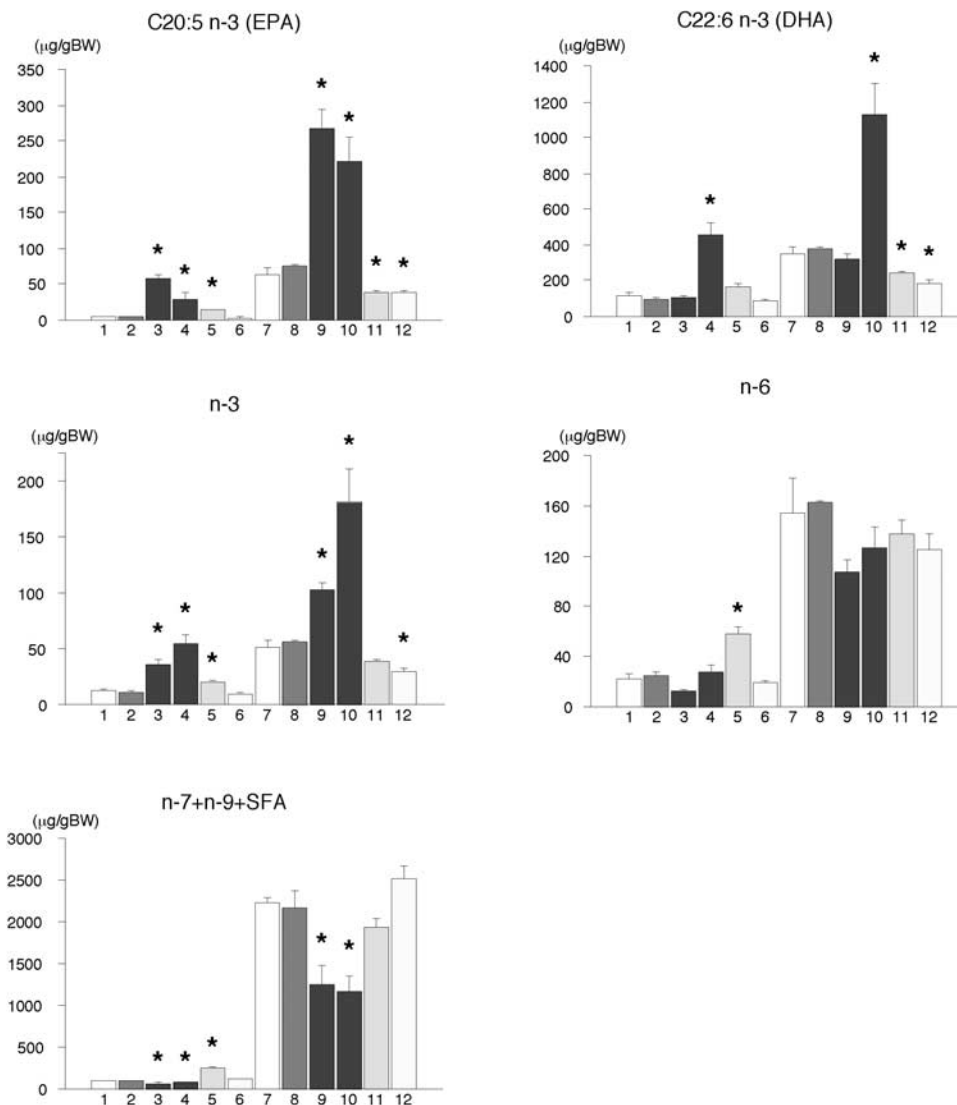


Fig. 5. Effects of PUFA on fatty acid composition in the liver of wild-type and *ob/ob* mice. The composition of fatty acids in liver was measured by gas chromatography. Based on the data shown in Table 2, n-3, n-6, or n-7/n-9/saturated fatty acids (SFA) content is calculated and illustrated. Diets are as follows: **lanes 1 and 7**, a high-carbohydrate, fat-free diet; **lanes 2 and 8**, a high-carbohydrate diet with 15% triolein (C18:1 n-9); **lanes 3 and 9**, 5% EPA ethyl ester (C20:5 n-3) plus 15% triolein (C18:1 n-9); **lanes 4 and 10**, 20% tuna fish oil; **lanes 5 and 11**, 0.125% fenofibrate, a PPAR $\alpha$  agonist; and **lanes 6 and 12**, 0.05% pioglitazone, a PPAR $\gamma$  agonist. **Lanes 1 to 6** are data from wild-type mice, and **lanes 7 to 12** are from *ob/ob* mice ( $n = 4$  for each group). **Bars** = SE. \*Denotes significance vs. control (**lane 1** or **7**) at  $P < .05$  (Student's  $t$  test). Series of experiments were performed twice for the same number of animals, and the data from a representative experiment are shown.

## Discussion

The current study clearly demonstrates that PUFAs reduce mature SREBP-1 and ameliorate hepatic steatosis in *ob/ob* mice. Based on our previous findings that PUFAs suppress SREBP-1<sup>10</sup> and that SREBP-1-null *ob/ob* mice have less triglycerides in liver,<sup>5</sup> we hypothesized that PUFA would decrease hepatic triglyceride storage through the reduction of SREBP-1. Our results provide a strong clinical implication that PUFAs are potentially useful for the treatment of hepatic steatosis. In addition, we provided further evidence that SREBP-1 regulates the storage of triglycerides in liver. In this regard, we speculate that the reduction of hepatic lipogenesis by PUFA resulted in a decrease in triglyceride storage. Two lines of evidence indicate decreased lipogenesis in PUFA-fed mouse livers: a decrease in lipogenic enzymes mRNA levels as shown by Northern blot analysis (Fig. 2) and the hepatic fatty acid composition (Table 2, Fig. 5), demon-

strating that PUFA administration decreased fatty acids such as oleate (C18:1 n-9), palmitate (C16:0), and palmitoleate (C16:1 n-7) that can be endogenously synthesized more profoundly than fatty acids such as linoleate (C18:2 n-6) that cannot be biosynthesized *de novo*.

In the present study, we show that EPA administration decreased hepatic triglyceride content in *ob/ob* mice, whereas it did not in a transgenic mouse model, which forcibly expressed a mature form of SREBP-1 protein in the liver (TgSREBP1). These findings indicate that the effect of EPA on the improvement of hepatic steatosis is mainly mediated by SREBP-1 reduction. In contrast, tuna oil, rich in DHA, was also effective in the TgSREBP1 mice. These data indicate that tuna oil may have additional positive effects beyond the suppression of SREBP-1. The difference between them might be derived from differences in composition and action of EPA versus DHA. In fact, although EPA can be converted to DHA



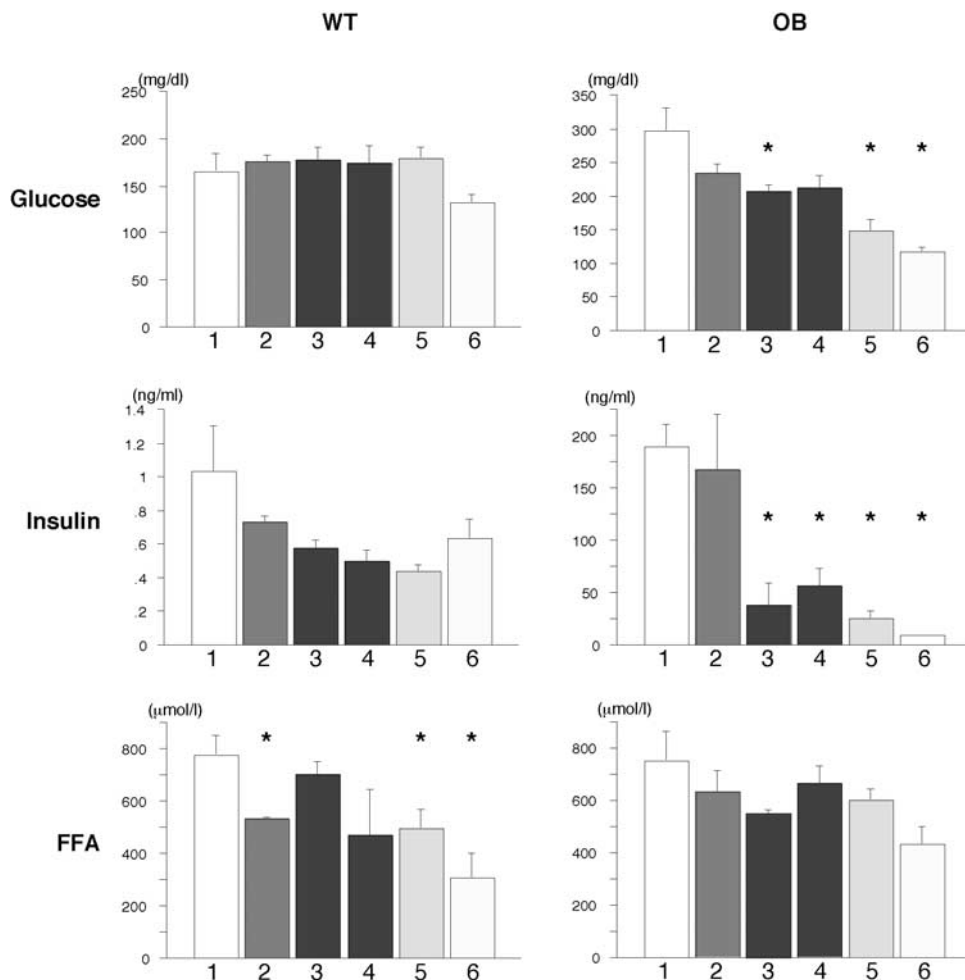


Fig. 6. PUFAs improve hyperglycemia and hyperinsulinemia of *ob/ob* mice. Plasma glucose, insulin, and free fatty acids (FFA) of wild-type (WT) and *ob/ob* (OB) mice ( $n = 4$  for each group) are shown. Diets are as follows: **lane 1**, a high-carbohydrate, fat-free diet; **lane 2**, 15% triolein (C18:1 n-9); **lane 3**, 5% EPA ethyl ester (C20:5 n-3) plus 15% triolein (C18:1 n-9); **lane 4**, 20% tuna fish oil; **lane 5**, 0.125% fenofibrate, a PPAR $\alpha$  agonist; **lane 6**, 0.05% pioglitazone, a PPAR $\gamma$  agonist. Bars = SE. \*Denotes significance vs. control (**lane 1**) at  $P < .05$  (Student's  $t$  test). Series of experiments were performed twice for the same number of animals, and the data from a representative experiment are shown.

endogenously with elongase and  $\Delta 6$ -desaturase via the Sprecher pathway,<sup>31</sup> the hepatic content of DHA in mice administered EPA was not elevated, as shown in Fig. 5, presumably because of the suppression of  $\Delta 6$ -desaturase expression by PUFA.<sup>24,25</sup> Although EPA and DHA are considered equally effective as suppressors of SREBP-1,<sup>10</sup>

the mechanisms by which PUFAs suppress triglyceride synthesis are known to be divergent,<sup>32</sup> and they might have different potency for the SREBP-1-independent pathways. It is also possible that some unknown substance in tuna oil could be responsible for the SREBP-1-independent action.

We showed that PUFA not only ameliorated hepatic steatosis but also attenuated insulin resistance in *ob/ob* mice. Although the precise mechanisms are currently unknown, we are speculating that this effect of PUFA was mediated at least partially by the PPAR $\alpha$  receptor because PUFAs are well known PPAR $\alpha$  ligands,<sup>27</sup> and a PPAR $\alpha$  agonist, fenofibrate, was also demonstrated to improve insulin sensitivity in *ob/ob* mice as shown in Fig. 6. We presume that this effect of PUFA is not mediated by SREBP-1 because it was previously shown that the disruption of SREBP-1 in *ob/ob* mice did not influence their insulin resistance.<sup>5</sup> However, it should be noted that EPA was equally competent in improving the hyperinsulinemia of *ob/ob* mice, whereas EPA was much weaker in PPAR $\alpha$  activation as estimated by Northern blot analysis

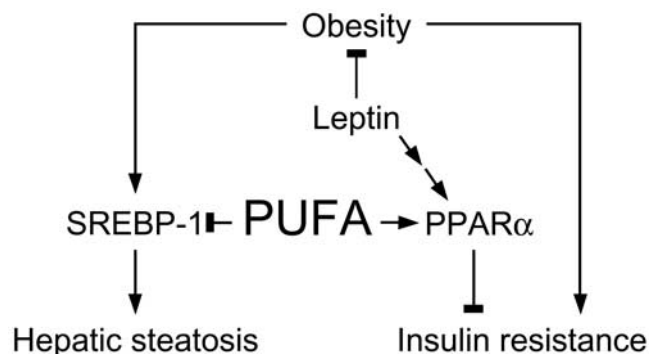


Fig. 7. Schematic representation of PUFA effects on obesity. PUFAs suppress SREBP-1 and activate PPAR $\alpha$ , thereby preventing excess triglyceride accumulation and ameliorating obesity-associated symptoms, including hepatic steatosis and insulin resistance.

for PPAR $\alpha$  target genes. It is tempting to speculate that there could also be other mechanisms for insulin sensitization by PUFA than the PPAR $\alpha$  pathway; for instance, it is possible that altered phospholipid composition and thereby membrane fluidity might be involved.<sup>33,34</sup>

We also showed that PUFAs significantly ( $P < .05$ ) reduce hepatic cholesterol content in wild-type mice (Fig. 4A). This is in accordance with previous reports<sup>35,36</sup> that fish oil feeding decreased hepatic cholesterol content in rodents. The mechanism by which this occurs is currently unknown, but it was documented that EPA has an inhibitory effect on hepatic cholesterol biosynthesis and an enhancing effect on hepatic biliary secretion.<sup>37</sup> Although the mRNA expression levels of cholesterol biosynthetic genes were not shown to be lowered by PUFA in Northern blotting (Fig. 2), these analyses do not account for possible posttranscriptional effects.

As we summarized in Fig. 7, PUFAs down-regulate SREBP-1 and, thereby, triglyceride synthesis and at the same time activate the PPAR $\alpha$  receptor, leading to the increased  $\beta$ -oxidation of fatty acids. Hence, PUFA can shift the energy balance from storage to consumption. The antilipogenic action of PUFA through SREBP-1 suppression has been proven to be independent of the PPAR $\alpha$  pathway by previous studies using PPAR $\alpha$ -knockout mice.<sup>28</sup>

From a therapeutic point of view, it was notable that PUFA also decreased plasma ALT levels. This means that PUFAs attenuate the liver injury resulting from excess triglyceride deposition. Although the precise pathophysiology as to how hepatic steatosis leads to steatohepatitis and even to cirrhosis remains unclear, liver disease might be a major contributor to overall obesity-related morbidity and mortality.<sup>38</sup> Our data suggest that PUFA might be of clinical use to prevent the liver dysfunction associated with over nutrition.

In conclusion, we demonstrate that PUFAs ameliorate hepatic steatosis through the suppression of SREBP-1 in *ob/ob* mice. Furthermore, PUFAs were suggested to be presumably of clinical value as a therapeutic drug for insulin resistance.

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