A Crucial Role of Sterol Regulatory Element-binding Protein-1 in the Regulation of Lipogenic Gene Expression by Polyunsaturated Fatty Acids*

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Dietary polyunsaturated fatty acids (PUFA) are negative regulators of hepatic lipogenesis that exert their effects primarily at the level of transcription. Sterol regulatory element-binding proteins (SREBPs) are transcription factors responsible for the regulation of cholesterol, fatty acid, and triglyceride synthesis. In particular, SREBP-1 is known to play a crucial role in the regulation of lipogenic gene expression in the liver. To explore the possible involvement of SREBP-1 in the suppression of hepatic lipogenesis by PUFA, we challenged wild-type mice and transgenic mice overexpressing a mature form of SREBP-1 in the liver with dietary PUFA. In the liver of wild-type mice, dietary PUFA drastically decreased the mature, cleaved form of SREBP-1 protein in the nucleus, whereas the precursor, uncleaved form in the membranes was not suppressed. The decreases in mature SREBP-1 paralleled those in mRNAs for lipogenic enzymes such as fatty acid synthase and acetyl-CoA carboxylase. In the transgenic mice, dietary PUFA did not reduce the amount of transgenic SREBP-1 protein, excluding the possibility that PUFA accelerated the degradation of mature SREBP-1. The resulting sustained expression of mature SREBP-1 almost completely canceled the suppression of lipogenic gene expression by PUFA in the SREBP-1 transgenic mice. These results demonstrate that the suppressive effect of PUFA on lipogenic enzyme genes in the liver is caused by a decrease in the mature form of SREBP-1 protein, which is presumably due to the reduced cleavage of SREBP-1 precursor protein.

The liver, the principal lipogenic organ, is responsible for the conversion of excess dietary carbohydrates to triglycerides. A high carbohydrate diet induces the synthesis of several lipogenic and glycolytic enzymes including acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase, ATP citrate lyase, malic enzyme, glucose-6-phosphate dehydrogenase, and pyruvate kinase (PK) (1–3). The regulation by PUFA has been shown to be primarily at the transcriptional level; however, the precise mechanism for this action remains unknown (5–7).

Sterol regulatory element-binding proteins (SREBPs) are transcription factors that belong to the basic helix-loop-helix-leucine zipper family and regulate enzymes responsible for the synthesis of cholesterol, fatty acids, and triglycerides (8). Unlike other members of the basic helix-loop-helix-leucine zipper family, SREBPs are synthesized as precursors bound to the endoplasmic reticulum and nuclear envelope. Upon activation, SREBPs are released from the membrane into the nucleus as a mature protein by a sequential two-step cleavage process. To date, three SREBP isoforms, SREBP-1a, -1c and -2, have been identified and characterized. The predominant SREBP-1 isoform in the liver is SREBP-1c. Whereas SREBP-2 is relatively selective in transcriptionally activating cholesterol biosynthetic genes, SREBP-1c has a greater role in regulating fatty acid synthesis than cholesterol synthesis in the liver (9–11, 30).

The role of SREBP-1 for the regulation of hepatic lipogenesis has been recently established. Changes in hepatic mature SREBP-1c protein levels were shown to parallel those of mRNAs for lipogenic genes in the liver using a dietary manipulation and a transgenic technology (12). Moreover, SREBP-1 has been demonstrated to be crucial for the carbohydrate stimulation of lipogenic genes in mice with a targeted disruption of SREBP-1 (30).

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These findings led us to hypothesize that the suppressive effect of PUFA on lipogenic gene transcription in liver is mediated through a decrease in mature SREBP-1 levels. To verify this hypothesis, the current studies were designed using wild-type mice and transgenic mice overexpressing mature SREBP-1c in the liver (TgSREBP-1c). First, we show that the amount of mature SREBP-1 in the liver is decreased by dietary PUFA. Next, we demonstrate that the expression of lipogenic genes in the liver of TgSREBP-1c mice is maintained at a relatively high level and that this expression follows the pattern of SREBP-1 levels that remain elevated even in the presence of dietary PUFA. These results indicate that the suppression of hepatic gene expression of lipogenic enzymes by PUFA is most likely due to the decrease in the mature SREBP-1 protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Triolein (95% grade) and ethyl linoleate were purchased from Sigma, and tristearin and casein were purchased from Wako Pure Chemical Industries (Osaka, Japan). Eicosapentaenoic acid (EPA) ethyl ester (96% grade) was provided by Meijide Pharmaceutical (Tokyo, Japan), fenofibrate by Laboratoires Fournier (Paris, France), and troglitazone by Sankyo Pharmaceutical (Tokyo, Japan). Fish oils (sardine and tuna) were provided by NOF (Tokyo, Japan). Standard laboratory chow, high carbohydrate fat-free diet (70% sucrose and 20% casein supplemented with methionine, vitamins, and minerals), and high protein fat-free diet (90% casein and no carbohydrate with methionine, vitamins, and minerals) were obtained from Oriental Yeast (Tokyo, Japan).

**Animals and Diets**—7-week-old male C57BL/6J mice (21–23 g) were purchased from CLEA (Tokyo, Japan) and adapted to the environment for 1 week prior to study. SREBP-1c transgenic mice overexpressing amino acids 1–436 of human SREBP-1c under control of the rat PEPCK promoter (TgSREBP-1c) were made as described previously (9). A line homozygous for the transgene was established. All mice were housed in a controlled environment with a 12-h light/dark cycle and free access to water and diet. Prior to sacrifice, each group of three animals was fed a diet containing the indicated fatty acids, prepared fresh daily, for 7 days. The diet for wild-type mice consisted of a high carbohydrate fat-free chow supplemented with 20% (w/w) tristearin, 20% triolein, 20% fish oil (sardine or tuna), 5% linoel acid, or 5% EPA ethyl ester. The diet for TgSREBP-1c mice was a high protein diet mixed with 20% triolein, 20% triolein plus 5% EPA ethyl ester, or 20% fish oil (sardine). For the comparison of high protein and high carbohydrate diets, wild-type mice were examined on diets composed of high protein or high carbohydrate chow mixed with 20% triolein or 20% fish oil (sardine). All mice were sacrificed during the early phase of the light cycle in a nonfasted state.

**Immunoblotting of SREBP Proteins**—Nuclear extracts and membrane fractions from mice livers were prepared as described previously (13). Aliquots of nuclear (20 µg) and membrane (30 µg) proteins were subjected to SDS-polyacrylamide gel electrophoresis. Immunoblot analysis was performed using the ECL Western Blotting Detection System kit (Amersham Pharmacia Biotec) and exposed to Eastman Kodak Co. XAR-5 film. The primary antibodies (polyclonal) were as described previously (14, 15).

**Northern Blotting**—Total hepatic RNA was isolated with Trizol reagent (Life Technologies), and 10-µg RNA samples (equally pooled from three mice) were run on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane. The cDNA probes used were cloned as described previously (15, 30). The probes were labeled with [α-32P]dCTP using the Megaprime DNA Labelling System kit (Amersham Pharmacia Biotec). The membranes were hybridized with the radiolabeled probe in Rapid-hyb Buffer (Amersham Pharmacia Biotec) at 65 °C and washed in 0.1× SSC, 0.1% SDS at 65 °C. Blots were exposed to Kodak XAR-5 film.

**RESULTS**

The Effect of PUFA on the Mature Form of SREBP-1 Protein in Wild-type Mice Livers—To examine the effect of PUFA on SREBP-1 in mice livers, wild-type mice were fed a high carbohydrate diet supplemented with PUFA (linoleate, EPA, or two kinds of fish oil rich in EPA or docosahexaenoic acid) for 7 days. Immunoblot analysis of liver nuclear extracts from these mice showed that feeding wild-type mice a diet with EPA for 7 days resulted in a ~3-fold decrease in the amount of hepatic mature SREBP-1 protein, compared with feeding with the control diet (high carbohydrate without fat). This suppressive effect of EPA on the mature SREBP-1 was consistently observed in six independent experiments including Figs. 1, a, b, and c, 2, and 7. Fish oil (sardine, rich in EPA, or tuna, rich in docosahexaenoic acid) decreased mature SREBP-1 protein more profoundly, as shown in Figs. 1, b and c, 2, and 7. Dietary linoleate also suppressed mature SREBP-1 to a lesser extent (Fig. 2). In contrast, neither saturated (tristearin) nor monounsaturated (triolein) fatty acids reduced mature SREBP-1 (Fig. 2). The amount of mature SREBP-2 protein did not change significantly with any of the dietary manipulations (Figs. 1, a and b, 2, 6a, and 7).

**Posttranslational Regulation of SREBP-1 by PUFA**—The generation of mature SREBP-1 protein requires several steps including transcription, translation, and a proteolytic cleavage from microsomal membranes. To clarify the mechanism by which PUFA down-regulates mature SREBP-1 protein, the amounts of SREBP-1 (both precursor and mature forms in the membrane and nuclear extracts, respectively; Fig. 1c) as well as SREBP-1 mRNA levels (Fig. 3) were compared between control and PUFA (EPA or fish oil)-fed mice. In contrast to the mature protein, which was profoundly decreased by PUFA feeding, no significant reduction was observed in either mRNA or membrane-bound precursor protein levels. This indicates that PUFA regulates the abundance of mature SREBP-1 protein mainly at a posttranslational level, presumably through cleavage and/or degradative processes.

**Suppression of Lipogenic Gene Expression by PUFA**—We compared mRNA levels of genes encoding lipogenic enzymes in the liver of mice fed diets with or without PUFA (linoleate, EPA, or fish oil) for 7 days as measured by Northern blot analysis. Consistent with previous reports, dietary PUFA suppressed hepatic expression of lipogenic genes such as ACC, FAS, stearoyl-CoA desaturase 1, glycerol-3-phosphate acyltransferase, ATP citrate lyase, malic enzyme, glucose-6-phosphate dehydrogenase, PK, and Spot 14 (Fig. 3). This down-regulation of lipogenic genes was confined to PUFA (linoleate, EPA, fish oil)-fed groups of mice and was not observed in mice fed saturated (i.e. tristearin) or monounsaturated (i.e. triolein)
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FIG. 2. Immunoblot analysis of SREBP-1 and -2 in nuclear extracts from livers of mice fed a high carbohydrate diet containing various fatty acids. Mice (three male C57BL/6J, 8 weeks old) were fed a high carbohydrate fat-free diet (lane 1) or a high carbohydrate diet supplemented with 20% tristearin (lane 2), 20% triolein (lane 3), 5% linoleate ethyl ester (lane 4), 5% EPA ethyl ester (lane 5), 20% sardine fish oil (lane 6), or 20% tuna fish oil (lane 7) for 7 days and sacrificed in a nonfasted state. Aliquots of nuclear extracts (20 μg of protein) from pooled livers of each group were subjected to immunoblot with antibody against mouse SREBP-1 or -2.

FIG. 3. Northern blot analysis of lipogenic and glycolytic enzymes from livers of mice fed a diet containing various fatty acids. Mice (three male C57BL/6J, 8 weeks old) were fed the indicated diet for 7 days and sacrificed in a nonfasted state. Diets were as follows. Lane 1, a high carbohydrate fat-free diet; lane 2, a high carbohydrate diet with 20% tristearin (18:0); lane 3, 20% triolein (18:1); lane 4, 5% linoleate ethyl ester (18:2); lane 5, 5% EPA ethyl ester (20:5); lane 6, 20% sardine fish oil; lane 7, 20% tuna fish oil. Total RNA (10 μg) pooled equally from three mice was subjected to Northern blotting, followed by hybridization with the indicated cDNA probes. A cDNA probe for 36B4 (acidic ribosomal phosphoprotein P0) was used to confirm equal loading. SCD1, stearoyl-CoA desaturase 1; GPAT, glycerol-3-phosphate acyltransferase; ACL, ATP citrate lyase; ME, malic enzyme; G6PD, glucose-6-phosphate dehydrogenase; S14, Spot 14.

The Effects of PUFA on the Mature Form of SREBP-1 and Lipogenic Gene Expression in TgSREBP-1c Mice Livers—To examine whether the PUFA suppression of lipogenic genes was ascribed to the decrease in mature SREBP-1, a transgenic mouse model that forcibly expresses a mature form of SREBP-1 protein in the liver (TgSREBP-1c) was used. In these transgenic mice, the expression of a nuclear form of SREBP-1c that is active without cleavage is under control of the rat PEPECK promoter and is induced when animals are fed a high protein, low carbohydrate diet (9). As shown in Fig. 4a, the mature SREBP-1 in the liver of TgSREBP-1c mice fed a high protein diet is dominated by the transgene product, the amount of which was comparable with that of intrinsic SREBP-1 mature form in wild-type mice on a high carbohydrate diet (Fig. 4a, lanes 1 and 2). The hepatic mRNA levels of lipogenic genes in these TgSREBP-1c mice were also similar to those in wild-type mice on a high carbohydrate diet (Fig. 5; lanes 1 and 2).

The effects of PUFA on the amount of mature SREBP-1 and the mRNA levels of lipogenic genes were examined in these transgenic mice. It was found that the amount of the transgene product was not affected by dietary PUFA (EPA or fish oil) (Fig. 4a). Furthermore, dietary PUFA did not change the mRNA levels of lipogenic genes such as ACC, FAS, stearoyl-CoA desaturase 1, glycerol-3-phosphate acyltransferase, ATP citrate lyase, malic enzyme, glucose-6-phosphate dehydrogenase, Spot 14, or PK in the liver of TgSREBP-1c mice (Fig. 5). These results demonstrate that the suppressive effect of PUFA on lipogenic genes is primarily mediated through the decrease in the amount of mature SREBP-1.

The Comparison of Protein and Carbohydrate-based Diets—To confirm that dietary PUFA suppresses mature SREBP-1 and lipogenic genes in mice fed protein-based diets in the same way as in mice placed on carbohydrate-based diets, wild-type mice were fed a high protein diet supplemented with triolein or fish oil for 7 days, and the amount of mature SREBP-1 (Fig. 6a) and the mRNA levels of lipogenic genes (Fig. 6b) in the liver were evaluated by immunoblot and Northern blotting analyses, respectively. As expected, dietary PUFA exerted the same suppressive effect on the background of high protein diets as was observed on high carbohydrate diets. It was also revealed that a high carbohydrate diet elevated mature SREBP-1 as well as mRNAs for lipogenic genes more strongly than a high protein diet. These results provide further evidence for the dependence of lipogenic gene transcription on mature SREBP-1.
The Effect of Peroxisome Proliferator-activated Receptor α (PPARα) Ligand on Mature SREBP-1—It is well known that PUFA are ligands for PPARα (16, 17), and some biological effects of PUFA such as induction of peroxisomal and microsomal fatty acid oxidation are mediated by PPARα (18). To determine whether PPARα is involved in the suppression of mature SREBP-1 by PUFA, the amount of mature SREBP-1 in wild-type mice fed a diet containing fenofibrate, a ligand for PPARα, for 7 days was examined. Immunoblot analysis of liver nuclear extracts showed that neither fenofibrate nor troglitazone, a ligand for PPARγ, affected the amount of mature SREBP-1 in the liver (Fig. 7). These data suggest that the suppressive effect of PUFA on mature SREBP-1 is not mediated by PPARα or -γ. The expected effects of PPARα were confirmed by the observation of increased mRNAs of acyl-CoA oxidase and cytochrome P-450 4A2 (data not shown).

DISCUSSION

In the present study, we clearly showed that the suppression of lipogenic gene expression by PUFA in the liver was primarily due to decreases in the mature form of SREBP-1. We first demonstrated that the abundance of mature SREBP-1 in the liver was decreased by dietary PUFA. A similar observation has been reported in a study in cultured cells (19). However, it was reported that mature SREBP-1 was also decreased by oleate, a monounsaturated fatty acid, which exhibited no significant effect on hepatic SREBP-1 in our in vivo study. Extensive in vivo studies using mouse or rat liver have demonstrated that an antilipogenic effect is confined to PUFA and that this response appears to be specific to liver (20). Our results describing the effects of different fatty acids on SREBP-1 nuclear protein in the liver are consistent with these previous reports. The cause for the discrepancy in the effect of oleate between liver and cultured cells is currently unknown.

As shown in Fig. 1c, we demonstrate that PUFA regulates the abundance of mature SREBP-1 protein primarily at the posttranslational level. Moreover, we show that PUFA did not decrease the amount of mature SREBP-1 directly expressed from the transgene, indicating that PUFA does not accelerate the degradation of SREBP-1 mature protein. These data suggest that the regulation of SREBP-1 by PUFA occurs at the step of cleavage of the precursor protein from membranes.

If the suppression of lipogenic gene expression by PUFA is caused by the decrease in mature SREBP-1 protein, sustained SREBP-1 expression should abolish the effect of PUFA. To verify this hypothesis, homozygous TgSREBP-1c mice fed a high protein diet were used. As described previously, the rat PEPCK promoter used for the transgene is activated by feeding a high protein, low carbohydrate diet (21). Consequently, the hepatic expression of mature SREBP-1 in these mice was approximately equal to that of carbohydrate-stimulated wild-type mice and was not affected by PUFA. In the presence of sus-
tained SREBP-1 mature protein levels, the expression of lipogenic genes in the liver of TgSREBP-1c mice was maintained as high as that of wild-type mice on a high carbohydrate diet irrespective of PUFA ingestion. This indicates that the mature form of SREBP-1 is a determining factor in the suppressive effect of dietary PUFA on lipogenic gene expression.

It should be noted that the mRNA of PK was expressed more strongly in wild-type mice fed a high carbohydrate diet than in TgSREBP-1c mice fed a high protein diet, although similar amounts of mature SREBP-1 were present. This observation suggests the possibility that the transcriptional regulation of the PK gene is more strongly controlled by transcription factor(s) other than SREBP-1. This notion could explain the weaker effect of PUFA on the mRNA level of PK. It is also consistent with the previous observation that SREBP-1 disruption had a smaller influence on mRNA expression of PK in comparison with those of other lipogenic enzymes whose induction by a high carbohydrate diet was completely abolished in the SREBP-1 knockout mice.

The cis-acting elements in the promoter region for carbohydrate stimulation and PUFA suppression of lipogenic genes have been analyzed by many investigators. In the case of enzymes such as FAS (22), ATP citrate lyase (23), and PK (24), glucose/insulin response elements overlap with PUFA response regions. Especially, the glucose/insulin and PUFA response element in the FAS promoter has been shown to contain an SREBP-binding site (25). The PUFA response region in the mouse stearoyl-CoA desaturase 1 promoter is also reported to have an SREBP-binding site (26, 27). These data are supportive of our finding that carbohydrate stimulation and PUFA suppression are mediated by a common molecule, SREBP-1. In contrast, promoter analyses of the Spot 14 gene previously revealed that the PUFA-regulatory region was located separately from the region for dietary carbohydrate and insulin responses (5).

Since PUFAs are known as activators of PPARα (16, 17), the effects of PUFA through PPARα were also examined. The finding that fenofibrate did not affect the amount of mature SREBP-1 in the liver indicates that the suppressive effect of PUFA on mature SREBP-1 is not mediated by PPARα. This finding is compatible with the previous study using PPARα-null mice, which showed that PPARα was not required for the PUFA-mediated inhibition of either FAS or Spot 14 gene expression (18).

It has now been established that SREBP-1 and SREBP-2 function to regulate fatty acid synthesis and cholesterol synthesis, respectively, with some overlap in function (9, 10). Since SREBP-2 is a regulator of the cholesterol biosynthetic pathway, it is itself highly controlled by cellular sterol levels. This regulation, including the actions of SREBP cleavage-activating protein (SCAP) and the site 1 and 2 proteases, has been extensively studied (8, 28, 29). However, the more fine tuned regulation of SREBP-1 has yet to be determined. The data presented here indicate for the first time that the cleavage of SREBP-1 in the liver could be regulated by a mechanism other than sterol concentrations and presumably in a fashion related to fatty acid metabolism. Further studies are needed to clarify the mechanism by which PUFA modulates the cleavage of SREBP-1 precursor protein in terms of the regulation of hepatic lipogenesis.

In summary, we have demonstrated that dietary PUFA decreased the amount of hepatic SREBP-1 mature protein by a reduction in the cleavage of SREBP-1 precursor protein, causing the suppression of lipogenic gene expression in the liver.