Polyunsaturated Fatty Acid Suppression of Hepatic Fatty Acid Synthase and S14 Gene Expression Does Not Require Peroxisome Proliferator-activated Receptor α*

Dietary polyunsaturated fatty acids (PUFA) induce hepatic peroxisomal and microsomal fatty acid oxidation and suppress lipogenic gene expression. The peroxisome proliferator-activated receptor α (PPARα) has been implicated as a mediator of fatty acid effects on gene transcription. This report uses the PPARα-deficient mouse to examine the role of PPARα in the PUFA regulation of mRNAs encoding hepatic lipogenic (fatty acid synthase (FAS) and the S14 protein (S14)), microsomal (cytochrome P450 4A2 (CYP4A2)), and peroxisomal (acyl-CoA oxidase (AOX)) enzymes. PUFA ingestion induced mRNA_FAS (2.3-fold) and mRNA_CYP4A2 (8-fold) and suppressed mRNA_AOX and mRNA_S14, by ≥80% in wild type mice. In PPARα-deficient mice, PUFA did not induce mRNA_AOX or mRNA_CYP4A2, indicating a requirement for PPARα in the PUFA-mediated induction of these enzymes. However, PUFA still suppressed mRNA_AOX and mRNA_S14 in the PPARα-deficient mice. These studies provide evidence for two distinct pathways for PUFA control of hepatic lipid metabolism. One requires PPARα and is involved in regulating peroxisomal and microsomal enzymes. The other pathway does not require PPARα and is involved in the PUFA-mediated suppression of lipogenic gene expression.

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The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; AOX, acyl-CoA oxidase; RXR, retinoid X receptor; FAS, fatty acid synthase; CYP4A2, cytochrome P450 4A2; PPRE, peroxisome proliferator response element; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction.
**Materials and Methods**

**Animals and Diets—** Male Sprague-Dawley rats (125–150 g) were obtained from Charles River Breeding Laboratories (Kalamazoo, MI). Male C57BL/6N × Sv129 mice (25–35 g), Fb homozygote wild-type (+/+) or knockout (−/−) were used for all of the feeding studies (40). Rats and mice were maintained on Teklad chow diet. In all feeding studies, rats and mice were meal-trained to a high carbohydrate diet as described previously (16, 17). The test diets consisted of a high carbohydrate (58% glucose) diet (ICN, Cleveland, OH) supplemented with either 10% (w/w) of complex fats (triolein, olive oil, fish (menhaden)), fatty acid ethyl esters (eicosapentaenoic acid or docosahexaenoic acid (Southeast Fisheries Science Center, Charleston, SC)), or 0.2% gemfibrozil (Sigma). All diets were supplemented with 0.1% butylated hydroxytoluene (17).

**RNA Analysis—** Total RNA from rat or mouse livers or from cultured rat primary hepatocytes was isolated using the guanidinium isothiocyanate procedure. The following cDNA probes were used to measure specific mRNAs: S14, pExoPEII6 (16); acyl-CoA oxidase, pTZ18R obtained from T. Osumi, Himeji Institute, Japan (41); fatty acid synthase, pFAS1 obtained from H. S. Sul, University of California-Berkeley (42); and β-actin, pRBA-1, obtained from L. Kedes, Stanford University, Palo Alto, CA.

The cDNA for CYP4A2 was cloned by differential display screening (43) of rat liver RNA. First strand cDNA was prepared (ΔDNA RNA Fingerprinting Kit, K1801–1, CLONTECH) from rat liver RNA of rats fed olive oil and fish oil for 5 days. PCR was performed using a Perkin-Elmer DNA thermal cycler 9600 as follows: one cycle of 94 °C, 10 s; 40 °C, 5 min; 68 °C, 5 min; two cycles of 94 °C, 5 s; 40 °C, 5 min, 68 °C, 5 min; 25 cycles of 94 °C 5 s; 60 °C, 1 min; and 68 °C, 2 min for 30 cycles of 94 °C, 10 s; 59 °C, 1 min; 72 °C, 2 min. 35S-labeled PCR products were separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. A band present in the fish oil derived cDNA and diminished in the olive oil derived cDNA was excised from the gel, replamified by PCR, and cloned into pGEM-T (Promega). The insert was sequenced by dideoxynucleotide sequencing (44). Computer assisted analysis (GCC, University of Wisconsin) of the cloned sequence with the GenBank database indicated that the cloned sequence was identical to a region of the rat cytochrome P450 4A2 sequence (accession no. 157719) at nucleotides 12624–12728 for the rat gene. This sequence was in the noncoding 3′-untranslated region. To obtain the sequence unique to CYP4A2 and containing the translated sequence, two PCR primers (5′-primer, 5′-atatatatgaactctggcccagggcccctcgtgg; 3′-primer, 5′-atatatatgactgctggggagtctctggattctgctgattg (underlined sequence represents EcoRI and Clal cloning sites, respectively) were synthesized. The primers represent the position of the nuclease in the rat CYP4A2 gene) were used to generate a cDNA from liver RNA of fish oil-fed rats by reverse transcriptase-PCR. The identity of the clone (pCYP4A2-2) was verified by DNA sequencing and used to measure CYP4A2 mRNA.

mRNA levels were measured by dot and Northern blot analyses (17) and the level of hybridization was quantified using a Molecular Dynamics PhosphorImager (Sunnyvale, CA) or by videodensitometry using an Agfa-2 scanner linked to a Macintosh computer with NIH Image software.

**Plasmid Construction and Primary Hepatocytes—** The construction of the reporter gene with the rat AOX PPRE fused upstream from the thymidine kinase promoter (TKCAT223) was described previously (38). Primary hepatocytes were obtained from rat liver by the collagenase perfusion method and transfected with specific DNAs in the presence of Lipofectin (16). Hepatocytes were treated with triiodothyronine along with specific fatty acids or peroxisome proliferators (WY14,643 or gemfibrozil) dissolved in Me2SO (16, 38). After 48 h of treatment, hepatocytes were analyzed for protein and CAT activity (38). CAT activity is defined as CAT units = counts/min of 35S-butylated chloramphenicol/h/100 µg of protein.

**Statistical Analysis—** All data are presented as the mean ± S.E. Statistical comparisons were made by a single-factor factorial analysis of variance using Microsoft Excel version 7.

**Results**

The Effects of Olive and Fish Oil on Hepatic Gene Expression in Wild-type (+/+ ) and PPARα Null (−/−) Mice—PPARα is the predominant PPAR subtype in rodent liver and has a central role in regulating the transcription of genes encoding hepatic peroxisomal and microsomal enzymes (24, 28, 40). To determine whether PPARα mediates PUFA regulation of hepatic gene expression wild type (+/+) and PPARα null (−/−) mice, were fed an olive oil or fish oil diet for 5 days. Northern analyses show that feeding (+/+) mice fish oil for 5 days resulted in a 2-fold (p < 0.0003) and 9-fold (p < 0.001) increase in hepatic mRNAs AOX and CYP4A2, respectively (Fig. 1, A and B). In contrast, fish oil did not significantly increase mRNAs AOX and CYP4A2 in the PPARα null (−/−) mice. These results indicate that PPARα is required for the PUFA-mediated induction of AOX and CYP4A2 mRNAs. While hepatic β-actin mRNA was elevated in the (−/−) mice when compared with the (+/+) mice, it was not affected by dietary manipulation.

Analysis of mRNAs encoding S14 and FAS shows both mRNAs were suppressed (20–70%) in both the wild type (+/+) and PPARα null (−/−) mice following fish oil feeding. Since
Preliminary studies have shown that PPARα is not required for the PUFA-mediated suppression of transcription of these genes.

The Effects of Olive Oil and Fish Oil on Hepatic AOX and S14 Gene Expression—Previous studies have shown that S14 and FAS are regulated by PUFA and peroxisomal proliferators in rat liver or primary hepatocytes (17, 45). The studies described below compare the PUFA and peroxisomal proliferator regulation of S14 and AOX in rat liver and primary hepatocytes. These in vivo and in vitro (rat primary hepatocytes) studies were performed to gain additional support for the idea that PUFA regulation of lipogenic gene expression is a result of a different pathway than PUFA regulation of AOX gene expression.

Rats were meal-fed diets supplemented with 10% olive oil, fish oil, eicosapentaenoic acid (20:5), or docosahexaenoic acid (22:6) for 5 days. When compared with chow-fed rats, hepatic mRNA_AOX is elevated ~40% in olive oil-fed rats and ~3-fold in fish oil-, 20:5-, and 22:6-fed rats (Fig. 2). mRNACyPA2 was induced >10-fold under similar conditions.2 While mRNA_S14 is induced ~2-fold by the olive oil feeding, fish oil, 20:5, and 22:6 suppressed mRNA_S14 by ~78%. Hepatic mRNA_FAS is also suppressed in fish oil-fed rats (17). Feeding mice (Fig. 1) or rats (Fig. 2) fish oil or their highly unsaturated fatty acid constituents (20:5 (n-3) or 22:6 (n-3)) leads to a pronounced induction of mRNA_AOX and mRNACyPA2 while inhibiting expression of mRNA_S14 and mRNA_FAS.

Time Course of Fish Oil and Gemfibrozil Effects on Hepatic S14 and AOX Gene Expression in Vivo—The rapidity of fish oil action on hepatic mRNA_AOX and mRNA_S14 was examined in rats fed fish oil for 1 to 5 days (Fig. 3A). Rats were meal-fed a high carbohydrate diet supplemented with 10% triolein oil for 10 days. Half of the rats were maintained on triolein oil, while the other half were switched to the fish oil diet. Both triolein- and fish oil-fed rats were killed after 1, 2, and 5 days. Total liver RNA was prepared and examined by dot-blot analysis for mRNA_AOX and mRNA_S14 levels; n = 4–10 per time point. These results are representative of two separate studies. Analysis of variance: a, 1-, 2-, and 5-day fish oil feeding on mRNA_AOX versus triolein oil fed mRNA_S14, p < 0.001; b, 2- and 5-day fish oil feeding on mRNA_AOX versus triolein oil fed mRNA_FAS, p < 0.001. B, after meal-training rats to a high carbohydrate fat-free diet, half of the rats were maintained on the high carbohydrate, fat-free diet, while the other half was switched to a diet supplemented with 0.2% (w/w) gemfibrozil for 4, 2, and 8 days. Dot-blot analysis was used to measure the mRNA_AOX and mRNA_S14 levels. The results were quantitated and normalized against the level of hepatic mRNA expressed in chow-fed rats, i.e. 1 unit. Analysis of variance: a, 0.001; b, 0.004; c, 0.002. These results are representative of two separate studies. The results were quantitated and normalized against the level of hepatic mRNA expressed in chow-fed rats, i.e. 1 unit; n = 3–4 per time point. These results are representative of two separate studies. Analysis of variance: a, 2, 4, and 8 days, gemfibrozil versus control diet fed, mRNA_AOX, p < 0.004; b, 4-day gemfibrozil fed versus control diet fed, mRNA_S14, p = 0.002.

FIG. 2. A comparison of the effect of olive oil and fish oil on hepatic S14 and AOX gene expression in vivo. Rats were meal-fed with diets supplemented with 10% (w/w) olive oil, menhaden (fish) oil, eicosapentaenoic acid (20:5), or docosahexaenoic acid (22:6) for 5 days. Total hepatic RNA was prepared and examined by dot-blot analysis for the effect of feeding on mRNA_AOX and mRNA_S14 levels. The results were quantified and normalized against the level of hepatic mRNA expressed in chow-fed rats, i.e. 1 unit. Analysis of variance for both mRNA_AOX and mRNA_S14, levels for menhaden oil-, 20:5-, and 22:6-fed versus olive oil-fed, p < 0.002. These results are representative of two separate studies; n = 4.

FIG. 3. Time course of fish oil and gemfibrozil effects on rat hepatic S14 and AOX gene expression. A, rats were meal-fed diets supplemented with 10% (w/w) triolein oil for 10 days. Half of the rats were maintained on triolein oil, while the other half were switched to the fish oil diet. Both triolein- and fish oil-fed rats were killed after 1, 2, and 5 days. Total liver RNA was prepared and examined by dot-blot analysis for mRNA_AOX and mRNA_S14 levels; n = 4–10 per time point. These results are representative of two separate studies. Analysis of variance: a, 1-, 2-, 4-, and 5-day fish oil feeding on mRNA_AOX versus triolein oil fed mRNA_S14, p < 0.001; b, 2- and 5-day fish oil feeding on mRNA_AOX versus triolein oil fed mRNA_FAS, p < 0.001. B, after meal-training rats to a high carbohydrate fat-free diet, half of the rats were maintained on the high carbohydrate, fat-free diet, while the other half was switched to a diet supplemented with 0.2% (w/w) gemfibrozil for 4, 2, and 8 days. Dot-blot analysis was used to measure the mRNA_AOX and mRNA_S14 levels. The results were quantitated and normalized against the level of hepatic mRNA expressed in chow-fed rats, i.e. 1 unit; n = 3–4 per time point. These results are representative of two separate studies. Analysis of variance: a, 2, 4, and 8 days, gemfibrozil versus control diet fed, mRNA_AOX, p < 0.004; b, 4-day gemfibrozil fed versus control diet fed, mRNA_S14, p = 0.002.

2 B. Ren, A. Thelen, and D. B. Jump, unpublished results.
have been reported previously (17). In contrast, mRNA_AOX remained unaffected after 1 day on the fish oil diet, yet was induced 2-3.5-fold after 2 and 5 days, respectively. Such results indicate that changes in S14 mRNA precede changes in AOX mRNA following initiation of fish oil feeding, but they do not argue against PPARα as a common mediator for the PUFA regulation of AOX and S14.

In an effort to separate the induction of AOX from the suppression of S14, the peroxisome proliferator, gemfibrozil was fed to rats at 0.2%/w/w for up to 8 days (Fig. 3B). mRNA_AOX was induced ~4-fold after 4 days on gemfibrozil, a level comparable to the level of mRNA_AOX after 5 days on fish oil. In contrast, gemfibrozil did not significantly suppress mRNA_S14 (Fig. 3B) or mRNA_FAS (not shown). Only a modest 22% inhibition of mRNA_S14 was seen after 4 days of gemfibrozil feeding. These results show that mRNAs encoding both S14 and AOX are affected by PUFA within 2 days of initiating fish oil feeding. However, the absence of a significant inhibition of mRNA_S14 following 8 days of gemfibrozil feeding argues against PPARα as a common mediator for PUFA regulation of both AOX and S14 gene expression.

**Effect of Fatty Acids on AOX and S14 Gene Expression in Primary Hepatocytes**—Primary hepatocytes provide a method to assess the direct effects of PUFA on hepatic gene expression (16, 17). To examine the effects of fatty acids on S14 and AOX mRNAs, primary rat hepatocytes were treated with albumin alone or albumin plus various fatty acids (Fig. 4). Treatment of primary hepatocytes with 18:1, 18:2, 18:3 (both n-3 and n-6), and 20:4 did not induce mRNA_AOX. Only 20:5 treatment induced mRNA_AOX (~2-fold). This finding is consistent with the effects of highly unsaturated fatty acids on AOX gene expression in vivo (Figs. 1 and 2). Oleic acid (18:1) did not affect S14 gene expression when compared with controls. However, 18:2

**DISCUSSION**

PPARα is the predominant PPAR subtype expressed in rat liver and it plays a central role in the induction of hepatic
peroxisomal and microsomal fatty acid oxidation (24, 40). Since several peroxisomal, microsomal and lipogenic enzymes are regulated by PUFA at the pretranslational level, we tested the hypothesis that dietary PUFA regulate hepatic fatty acid oxidation and de novo lipogenesis through a common mediator, i.e. PPARα. Interestingly, all studies reporting on fatty acid regulation of PPARα have been carried out by over expressing receptors in established cell lines. No studies have directly examined the role PPARα may have in fatty acid-regulated hepatic gene transcription. The PPARα-null mouse allows such an analysis. Coupling this genetic approach with other studies has allowed us to show for the first time that 1) PPARα is required for PUFA-mediated induction of hepatic mRNA AOX and mRNA CYP4A2 (Fig. 1); 2) PPARα is not required for PUFA-mediated suppression of mRNA S14 or mRNA FAS (Fig. 1); 3) while 18:2 (n-6), 18:3 (n-6 and n-3), 20:4 (n-6) and 20:5 (n-3) suppress mRNA S14 and mRNA FAS, only 20:5 (n-3) induces mRNA AOX in primary hepatocytes (Fig. 4); 4) while gemfibrozil induces hepatic mRNA AOX, it has little or no effect on mRNA S14 or mRNA FAS (Fig. 3). Taken together, these studies indicate that PUFAs of peroxisome/microsomal fatty acid oxidation and de novo lipogenesis in rat liver does not involve PPARα as a common mediator. The differential effect of specific fatty acids, i.e. 18:2, 18:3 (n-3 and n-6), 20:4 (n-6), versus gemfibrozil underscores the lack of coordinate regulation of these pathways in rat liver. Such studies indicate that PUFAs regulates at least two pathways in liver, one involves PPARα and controls expression of genes encoding proteins involved in peroxisomal and microsomal fatty acid oxidation. The other mechanism is PPARα-independent and is involved in the PUFA-mediated suppression of lipogenic gene expression.

PUFA suppress hepatic mRNA S14 and mRNA FAS levels by inhibiting gene transcription (15–17). From the data reported above, this inhibitory mechanism does not require PPARα. Although the mechanism of PUFA induction of hepatic mRNA AOX and mRNA CYP4A2 has not been established, the following studies implicate transcription as the principal mode of PUFA regulation of AOX and CYP4A: 1) peroxisomal proliferators rapidly induce transcription of genes encoding AOX, the bifunctional enzyme, thiolase and CYP4A subtypes 1–3 (26, 40); 2) PPARα is required for the induction of these genes (40); 3) PPARα binds PPREs as PPAR/RXR heterodimers in the promoters of these genes and stimulates transcription of cis-linked reporter genes (20–33, 38); 4) fatty acids activate PPARα and stimulate transcription of cis-linked reporter genes (20–33) (Fig. 5), and 5) PPARα is required for the PUFA induction of hepatic mRNA AOX and mRNA CYP4A2 (Fig. 1).

Previous efforts to examine the involvement of PPARα in PUFA regulation of lipogenic gene expression showed that the cis-regulatory targets for PUFA and PPAR in the S14 promoter (38, 45) did not converge. Analysis of stearyl-CoA desaturase 1 gene expression indicated that peroxisomal proliferators/PPAR induced by PUFA-suppressed transcription (46). Such studies argued against PPAR as a mediator of PUFA effects on lipogenic gene transcription. However, the over expression of receptors does not necessarily reflect physiologically relevant processes. The use of the PPARα-null mouse allows us to directly evaluate the role PPARα plays in PUFA regulation of hepatic gene expression. In contrast to (+/+) mice, hepatic mRNA AOX and mRNA CYP4A2 was not significantly induced in PPARα (−/−) mice by the PUFA diet indicating a requirement for PPARα in the PUFA-mediated induction of these enzymes. The fact that hepatic mRNA S14 and mRNA FAS was suppressed in both (+/+) and (−/−) mice provides strong evidence against a requirement for PPARα for PUFA-mediated suppression of S14 and FAS gene transcription. While these studies confirm our earlier suggestion that PPAR did not mediate PUFA suppression of S14 gene transcription, they provide new information on the requirement for PPARα in the PUFA-induction of AOX and CYP4A2 and the lack of involvement of PPARα in PUFA-mediated suppression of FAS gene transcription or L-pyruvate kinase gene expression. While other PPAR subtypes (PPARγ and PPARδ) (24, 39) are expressed in liver, Northern analyses suggests PPARγ and δ are minor subtypes in rodent liver. However, their role in PUFα control of hepatic gene expression cannot be excluded.

An important outcome of these studies is the finding that of all the PUFA tested, only 20:5 (n-3) activates PPARα in liver. Several groups have reported on fatty acid activation of PPARα in established cell lines like CV-1 and HeLa (20–24). Recently three groups reported that specific fatty acids, i.e. 18:2 (n-6), 18:3 (n-3 and n-6), and 20:4 (n-6) are ligands for PPARα (47–49). These same ligands do not activate PPARα or induce mRNA AOX in primary hepatocytes (Figs. 4 and 5). Feeding animals soybean or corn oil, oils containing 18:2 and 18:3 fatty acids, does not induce peroxisomal enzymes (4). This apparent conflict can be reconciled by the fact that primary hepatocytes have a high capacity for fatty acid oxidation, triglyceride synthesis and very low density lipoprotein secretion (7). We speculate that these pathways prevent intracellular fatty acids from accumulating to levels that activate PPARα. Interestingly, 20:5 (n-3) was the only PUFA tested here that activated PPARα. 20:5 (n-3) is reported to be poorly oxidized in mitochondria and poorly incorporated into complex lipids, such as triacylglycerides (7). Thus, 20:5 (n-3) might accumulate in the cell and mimic a state of fatty acid overload in the liver. Fatty acid overload resulting from high fat feeding (>50% calories as fat), uncompensated diabetes and liver disease have all been reported to increase peroxisomal β-oxidation (26, 50, 51). Alternatively, 20:5 (n-3) might be metabolized to an active ligand. Recent studies have suggested that the leukotriene, LTB4, is a ligand for PPARα (52). Indeed, LTB4 is derived from 20:4 (n-6) by the action of 5-lipoxygenase and LTA4 hydrolase. If this pathway were operative, we would expect 20:4 (n-6) treatment of hepatocytes to activate PPARα and induce mRNA AOX. The lack of a 20:4 (n-6) effect on mRNA AOX and PPARα along with the low LTA4 hydrolase activity associated with liver cells (53) suggest that LTB4 is not the active ligand for 20:5 (n-3) activation of PPARα. However, the in vitro model used in this work may lack factors present in the in vivo system.

In summary, PUFA induce peroxisomal and microsomal fatty acid oxidation and suppress de novo lipogenesis (2–15). This apparent coordinate regulation of lipid metabolism does not involve PPARα as a common mediator. While highly unsaturated n-3 fatty acids, such as 20:5, can activate PPARα resulting in increased mRNA AOX and mRNA CYP4A2, PPARα does not mediate the suppressive effects of 18:2, 18:3 (n-3 and n-6), 20:4 (n-6), or 20:5 (n-3) on lipogenic gene expression. Thus, PUFA suppression of S14 and FAS gene transcription is mediated by a pathway that is independent of PPARα. The underlying mechanism of this alternative pathway is currently under investigation.

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