Unsaturated Fatty Acid Regulation of Peroxisome Proliferator-activated Receptor α Activity in Rat Primary Hepatoctyes*

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Peroxisome proliferator-activated receptors (PPARs α, β, γ1, and γ2) are widely regarded as monitors of intracellular nonesterified fatty acid (NEFA) levels. As such, fatty acid binding to PPAR leads to changes in the transcription of many genes involved in lipid metabolism and storage. Although the composition of the intracellular NEFA pool is likely an important factor controlling PPAR activity, little information is available on factors affecting its composition. Accordingly, we have examined the effects of exogenous fatty acids on PPAR activity and NEFA pool composition in rat primary hepatocytes. Prior to the addition of fatty acids to primary hepatocytes, nonesterified unsaturated fatty acid levels are very low, representing ≤0.5% of the total fatty acid in the cell. The relative abundance of putative PPAR ligands in the NEFA pool is 20:4n-3 > 18:2n-6 = 18:1n-9 > 22:6n-3 > 18:3n-6/3-6 = 20:5n-3. Of these fatty acids, only 20:5n-3 and 22:6n-3 consistently induced PPARα activity. Metabolic labeling of primary hepatocytes indicated that both 14C-18:1n-9 and 14C-20:5n-3 are rapidly assimilated into neutral and polar lipids. Although the addition of 18:1n-9 had no effect on NEFA pool composition, 20:5n-3 mass increased >15-fold within 90 min. Changes in NEFA pool 20:5n-3 mass correlated with dynamic changes in the PPARα-regulated transcript mRNA CYP4A. Metabolic labeling also indicated that a significant fraction of 14C-20:5n-3 was elongated to 22:5n-3. Cells treated with 22:5n-3 or 22:6n-3 led to a significant accumulation of 20:5n-3 in the NEFA pool through a process that requires peroxisomal β-oxidation and fatty acyl CoA thioesterase activity. Further analyses suggest that 20:5n-3 and 22:6n-3, but not 22:5n-3, are active ligands for PPARα. These studies suggest that basal fatty acid levels in the NEFA pool coupled with rates of fatty acid esterification, elongation, desaturation, peroxisomal β-oxidation, and fatty acyl thioesterase activity are important determinants controlling NEFA pool composition and PPARα activity.

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The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; LBD, ligand-binding domain; NEFA, nonesterified fatty acids; PUFAs, polyunsaturated fatty acids; FABP, fatty acid-binding protein; DDC, diethylidithiocarbamate; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CYP, cytochrome P-450; RP, reverse phase; HPLC, high pressure liquid chromatography.

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supplied as saturated and monounsaturated fat are \( \leq 20\% \) of total calories, hepatic PPARα-regulated transcripts, like acyl CoA oxidase and cytochrome P-450 4A-4CYFPA4A are marginally affected (15, 16). Substituting fish oil, a rich source of 20- and 22-carbon N3-PUFA, leads to a pronounced induction of enzymes involved in lipid oxidation. Eicosapentaenoic acid (20:5n-3), a known PPARα ligand (1), induces acyl CoA oxidase and CYP4A mRNA levels in primary hepatocytes and activates a PPARα-reporter gene. Moreover, PUFAs induction of these transcripts requires a functional PPARα. However, other PPARα ligands, like 18:1n-9 and 20:4n-6, have little effect on PPARα activity or PPARα-regulated genes in cultured primary hepatocytes (16).

The differential effects of putative fatty acid ligands on PPARα activity and hepatic gene expression raise the question of the role hepatic metabolism plays in the control of PPARα activity. Accordingly, we have carried out a detailed analysis of fatty acid regulation of PPARα as well as fatty acid metabolism in primary hepatocytes. These studies will document dynamic changes in the intracellular NEFA pool composition and effects on PPARα-regulated hepatic gene expression. In addition, our studies will provide evidence for \( \beta \)-oxidation products of 22-carbon fatty acids in the NEFA pool and their effects on PPARα activity.

**MATERIALS AND METHODS**

**Primary Hepatocytes and Transfections—**Male Sprague-Dawley rats were maintained on a Tek-Lad chow diet ad lib and were used for primary hepatocyte preparation (17). For metabolic labeling and RNA studies, the cells were plated onto 50- or 100-mm Primaria plastic dishes at 3 \times 10^6 or 10^7 cells/plate, respectively, in Williams E with 10 mM lactate, 10 nM dexamethasone, 100 nM insulin, and 10% fetal calf serum. The medium was changed the next morning to Williams E with 10 mM lactate, 10 mM dexamethasone, and 100 nM insulin. For transfection studies, the cells were plated in the same medium onto 6-well Primaria plastic dishes at 10^6 cells/well. The cells were transfected in this serum-free medium using Lipofectin (6 \muLg DNA) or LipofectAMINE 2000 (1.3 \muLg DNA) (Invitrogen) as described (14, 18, 19). pmR-PPARα-LBD and TKMH100x-Luc were previously described (20). pmR-PPARα-LBD is a fusion of the Gal4-DNA-binding domain fused to the ligand-binding domain of rPPARα. The TKMH100x-Luc reporter contains four binding sites for the Gal4-DNA-binding domain. pRGG-Luc was obtained from Promega (Madison, WI) and served as a control for transfection efficiency as well as nonspecific effects on promoter activity. The medium was changed the next morning to Williams E with 25 mM glucose, 10 mM dexamethasone, 100 mM insulin, fatty acid (NuChek Prep, Elysian, MN), and bovine serum albumin (BSA to fatty acid ratio was 1:5) or the PPARα agonist, WY14,643 (Chemsyn Science Laboratories, Lenexa, KS). After 24 h of treatment, the cells were harvested for luciferase assays. Each treatment involves triplicate samples, and each study was repeated at least twice. The results are expressed as normalized luciferase activity: firefly luciferase activity/\( \beta \)-galactosidase luciferase activity.

**RNA Isolation and Northern Analysis—**Primary hepatocytes were plated onto 100-mm primary plates at 10^6 cells/plate and treated as described above. RNA was extracted from rat primary hepatocytes using Triazol (Invitrogen) and separated electrophoretically in denaturing agarose gels, transferred to nitrocellulose, and probed with \( ^{32}P\)-cDNA for CYP4A (16, 19). Levels of hybridization were quantified using a Molecular Dynamics Phosphorimager 820 (Amer sham Biosciences, Piscataway, NJ).

**Fatty Acid Metabolism—**Primary hepatocytes were plated in the same medium as described above but onto 50-mm Primaria plastic dishes at 5 \times 10^6 cells/plate. The ratio of culture medium to cell number was maintained constant for the different plating conditions. The cells were treated with different fatty acids (see figure legends for details) essentially as described for gene expression studies. The cells were incubated overnight in serum-free medium prior to fatty acid treatment. After metabolic labeling studies, the cells were treated with \( ^{14}C \)-labeled fatty acids in 3 ml of medium containing 250 \muCi fatty acid (0.5 \muCi, 1.7 Ci/mol) (20). \( ^{14}C\)-Labeled fatty acids were purchased from PerkinElmer Life Sciences. Nonradioactive fatty acids were purchased from NuChek Prep. Fatty acid-free BSA (Roche Applied Science) was included at a ratio of fatty acid to BSA of 5:1.

After fatty acid treatment, the medium was collected, the cells were washed one time with phosphate-buffered saline and 40 mM BSA, washed one time with phosphate-buffered saline, and resuspended in 500 \muL of 40% methanol (20). This washing method minimizes contamination of cellular lipids with unincorporated free fatty acids. The methanol extract of cells was acidified with HCl to 0.25 \%, and lipids were extracted with chloroform:methanol (2:1) containing 1 mM butyrate hydroxytoluene (BHT). The protein and aqueous phases were re-extracted with chloroform. The organic phases were pooled, dried under nitrogen, resuspended in chloroform and 1 mM BHT, and stored at \(-80^\circ \) C. \(^{14}C\)-Labeled lipid extracts were further fractionated by thin layer chromatography (LKB 6100 Silica 60: Whatman silica, dried, and hexane-diethyl ether-acetic acid (90:30:1). The distribution of \( ^{14}C\)-fatty acids in various lipid fractions was visualized by exposure of the TLC to a phosphorimaging screen (Amer sham Biosciences), and the levels of radioactivity were quantified. The location of lipids was compared with authentic standards for triacylglycerol, diacylglycerol, cholesterol ester, fatty acids, fatty acid (wax) esters, and glycerol- and sphingophospholipids (Avanti Polar Lipids). The uptake of \( ^{14}C\)-fatty acids into cells and the organic fraction was quantified by scintillation counting. The depletion of \( ^{14}C\)-labeled fatty acids from medium was quantified by scintillation counting and TLC followed by phosphorimaging analysis as described above.

The NEFA fraction in total cellular lipids was fractionated on aminopropyl columns (Alltech Associates, Deerfield, IL) (20). Lipid extracts in chloroform and 1 mM BHT were applied to aminopropyl columns (100 mg) and washed extensively with chloroform/isopropanol (2:1) to remove neutral lipids. NEFA were eluted with diethyl ether and 2% acetic acid. Phospholipids were retained on the column. The diethyl ether, 2% acetic acid fraction was dried under nitrogen, resuspended in methanol and 10 mM BHT, and used directly for RP-HPLC fractionation and quantification of unsaturated fatty acids. The fractional recovery of NEFA from whole cell lipid extracts was \( \geq 95\% \).

**RP-HPLC Analysis of Unsaturated Fatty Acids—**The total extracted lipids were saponified (0.4 % KOH in 80% methanol for 1 h at 50°C), neutralized, extracted in diethyl ether and 1% acetic acid, and resuspended in methanol and 0.1 mM BHT for RP-HPLC analysis (reverse phase C18 column, Symmetry Shield, 2487 UV detector set to 192 nm with a 600 Controller; Waters Corp., Milford, MA). A linear gradient of 22 to 100% acetonitrile and 0.1% acetic acid over 40 min was used to fractionate unsaturated fatty acids (20). Verification and quantification of unsaturated fatty acids by RP-HPLC used authentic fatty acid standards (NuChek Prep) and Win-flow Radio HPLC software (INUS Systems, Inc, Tampa, FL). The identity of specific fatty acids was verified by gas chromatography/mass spectrometry at the mass spectrometry facility at Michigan State University.

**RESULTS**

**Effect of Unsaturated Fatty Acids on PPARα Activity in Primary Hepatocytes—**To examine the effect of unsaturated fatty acids on PPARα activity in primary hepatocytes, the cells were transfected with a chimeric receptor composed of the PPARα ligand-binding domain (LBD) fused to a Gal4-DNA-binding domain. Co-transfection with the TKMH100x4Luc reporter allows for an examination of changes in PPARα activity without recruitment of its heterodimer partner, retinoid X receptor, a prospective target for fatty acid control (21).

Primary hepatocytes were treated with various unsaturated fatty acids at 250 \muM (Fig. 1A). The 18- and 20-carbon fatty acids bind PPARα in vitro with affinities (IC_{50}) ranging from 0.3 to 1.2 \muM (1). Binding affinities for the 22-carbon fatty acids, 22:5n-3 and 22:6n-3, have not been reported. Of the putative PPARα ligands tested, 20:5n-3 and 22:6n-3 consistently induced PPARα. The 18-carbon fatty acids 18:1n-9, 18:2n-6, and 18:3n-3 and the 20-carbon N6-PUFA, 20:4n-6 did not consistently affect PPARα activity. For comparison, WY14,643 induced PPARα activity nearly 6-fold.

A dose response analysis reveals the difference in effect of the two prospective ligands on PPARα activity (Fig. 1B). Increasing the dose of 20:5n-3 significantly induced PPARα activity (8-fold at 1 mM), whereas a comparable dose of 18:1n-9 had no effect. These studies confirm previous findings on the difference.
separate studies with triplicate samples per group. The insert for

was used to correct for transfection efficiency and to assess nonspecific

luciferase activity, as described above; means

throughout this study. After a 24-h treatment period, the cells were

fied in the figure. The fatty acid to BSA ratio was maintained at 5:1

n-9 or 20:5-n-3 on PPAR

primary hepatocytes (15, 16). These studies also indicate that

activators of PPAR

activity is due to its conversion to an active ligand, e.g. 20:5-n-3.

Analysis of the Hepatocellular Unsaturated Fatty Acid Mass prior to Fatty Acid Treatment—Numerous reports with a variety

cell lines indicate that a broad array of fatty acids affect

PPAR activity (2). In fact, our studies with the rat hepatoma cell line, FTO2B, indicates that, with the exception of 22:5-n-3, all of the fatty acids examined in Fig. 1 induce PPARn-2-fold.2

We hypothesized that the difference in fatty acid responsiveness of rat primary hepatocytes and established cell lines is related to the distribution of fatty acids in the NEFA pool. Accordingly, the mass of hepatocellular fatty acids in the total saponified lipid fraction and in the NEFA pool was examined. Our analysis focused only on unsaturated fatty acids (Fig. 2).

The abundance of total saponified and NEFA in primary hepatocytes shows that the prominent unsaturated fatty acids include 18:1-n-9, 18:2-n-6, and 20:4-n-6. Docosahexaenoic acid (22:6-n-3) is of intermediate abundance, whereas 18:3-n-3/6, 18:3-n-6, 20:5-n-3, 22:4-n-6, and 22:5-n-3 are low abundance unsaturated fatty acids in primary hepatocytes. The relative abundance of these unsaturated fatty acids is comparable with fatty acid profiles reported for liver (22, 23). Interestingly, the NEFA pool displays nearly the same relative abundance of fatty acids as seen in the total saponified lipid fraction, i.e. 18:1-n-9 = 18:2-n-6 = 20:6-n-3 > 22:6-n-3 > 18:3-n-3/6 = 20:5-n-3. Moreover, this profile is similar to the array of fatty acids bound to L-FABP (24). As expected, the mass of fatty acids in the NEFA pool is very low, representing <0.5% of the total saponified fatty acid level.

For comparison, 18:1-n-9 is the predominant fatty acid in the total saponified (480 nmol/mg protein) and NEFA (2.2 nmol/mg protein) fractions in FTO2B cells. All other unsaturated fatty acids are very low: <50 nmol/mg protein in the saponified fraction and <0.2 nmol/mg protein in the NEFA fraction.2 The profile of fatty acids in FTO2B cells parallels the distribution of fatty acids in the fetal calf serum used to maintain the cells. Thus, FTO2B cells display a very different fatty acid profile from primary hepatocytes.

Metabolic Labeling of Primary Hepatocytes—Because of the striking difference in effect of 18:1-n-9 and 20:5-n-3 on PPARn activity and the >100-fold difference in mass of 18:1-n-9 and

2 A. Pawar and D. B. Jump, unpublished observation.
The fraction of 14C-fatty acid recovered as NEFA is very low, representing ~1% of the total 14C-fatty acid in organic extracts. The addition of 14C-18:1n-9 to cells results in an accumulation of 18:1n-9 to ~1 nmol/mg protein between 1.5 and 6 h; this value was sustained up to 24 h. Interestingly, the total mass of 18:1n-9 in the NEFA pool remained unchanged (~1 nmol/mg protein) throughout the 24-h treatment period. Thus, the 18:1n-9 in the NEFA pool is composed predominantly of exogenous 18:1n-9. The lack of change in 18:1n-9 mass in the NEFA pool indicates that 18:1n-9 is rapidly esterified.

The addition of 14C-20:5n-3 to primary hepatocytes also increased to nearly 1 nmol/mg protein by 90 min but declined to ~0.5 nmol/mg protein by 24 h. Like 18:1n-9, the exogenous 20:5n-3 represents the major fraction of 20:5n-3 in the NEFA fraction. In contrast to 18:1n-9, 20:5n-3 mass in the NEFA pool is very low prior to addition of fatty acids to cells. Thus, the addition of 20:5n-3 to hepatocytes leads to a 17-fold increase in mass within 90 min.

Fig. 4C illustrates how fatty acid treatment perturbs the mass of unsaturated fatty acid in the NEFA pool. This figure illustrates the masses of 18:1n-9 (white bars) and 20:5n-3 (black bars) and the sum of other nonesterified unsaturated fatty acids (gray bars) in the NEFA pool. These other unsaturated fatty acids remained relatively constant at ~1 nmol/mg protein following the addition of either 18:1n-9 or 20:5n-3 to cells. Although the addition of 18:1n-9 does not perturb the total mass of unsaturated fatty acids, the addition of 20:5n-3 leads to an approximately 25% increase in the total nonesterified unsaturated fatty acid mass within 90 min. By 24 h, the total mass of all nonesterified unsaturated fatty acids declines to pretreatment levels (~2 nmol/mg protein) by 24 h.

The total mass of 20:5n-3 incorporated into the saponified lipid fraction increased from 5.5 to ~347 nmol/mg protein within 1.5 h of addition of 20:5n-3 to cells (not shown). The mass increase of 20:5n-3 in the NEFA pool represents ~0.28% of the total cellular 20:5n-3. This fraction is comparable with the level of 20:5n-3 in the NEFA pool relative to total cellular 20:5n-3 prior to fatty acid treatment (Fig. 2). Clearly, the major fraction of cellular 20:5n-3 is esterified and assimilated into complex lipids (Fig. 3). Because 18:1n-9 did not increase in the NEFA pool argues against contamination of these extracts with medium lipids. Whether the apparent enrichment of the NEFA pool is due to different rates of fatty acyl CoA formation or the return of 20:5n-3 to the NEFA pool by metabolic events remains unresolved.

Mass of 20:5n-3 in the NEFA Pool, and Not the Formation of Eicosanoids, Correlates with Induction of the PPARα-regulated Transcript, mRNA CYP4Aα.

To determine whether the changes in intracellular nonesterified 20:5n-3 (Fig. 4) correlated with effects on gene expression, we measured mRNA CYP4Aα, a
PPARα-regulated transcript (15, 16). Addition of 20:5n-3 to primary hepatocytes induced a prompt rise (2-fold within 6 h) in mRNA_CYP4A, followed by a 50% drop by 24 h (Fig. 5). The 20:5n-3-mediated induction of mRNA_CYP4A was blocked by the inhibitor of transcription, actinomycin D (not shown). The decline in mRNA_CYP4A after the 6-h time point likely represents diminished stimulation of transcription coupled with enhanced mRNA_CYP4A turnover. Vehicle and 18:1n-9-treated cells displayed a decline in mRNA_CYP4A over the 24-h treatment period. The profile of the 20:5n-3 effect on mRNA_CYP4A parallels the change in intracellular nonesterified 20:5n-3 (Fig. 4, B and C). Overall, the net gain in mRNA_CYP4A following 20:5n-3 addition is ~2-fold, which is comparable with the change in CYP4A protein (not shown). Taken together, these results reveal dynamic changes in 20:5n-3 within the NEFA pool, which correlate well to PPARα-regulated hepatic gene transcription. 

Eicosanoids, like leukotriene B4, have been reported to be PPARα ligands (2, 7–9). Previous studies suggested that inhibitors of cyclooxygenase and lipoxygenase had little impact on fatty acid-regulated hepatocyte gene expression (14). The addition of 20:5n-3 to primary hepatocytes leads to changes in levels of oxidized lipids in the NEFA pool (not shown). NADPH-dependent microsomal fatty acid oxidation represents a likely route for the generation of these oxidized lipids. To determine whether this pathway contributes to the 20:5n-3 control of gene expression, hepatocytes were treated with the inhibitor of microsomal oxidation, diethyldithiocarbamate (DDC) (27). Studies with isolated rat hepatic microsomal preparations indicated that DDC is a robust inhibitor of NADPH-dependent oxidation of both 20:4n-6 and 20:5n-3.2 Arachidonic acid typically has a

**Fig. 4.** Change of 18:1n-9 and 20:5n-3 in the NEFA pool following fatty acid challenge. Quantitation of 18:1n-9 (A) and 20:5n-3 (B) mass in the NEFA lipid fraction following treatment of primary hepatocytes with 14C-18:1n-9 or 14C-20:5n-3. The hepatocytes were treated with 18:1n-9 or 20:5n-3, the total lipids were separated by TLC, and the NEFA fraction was quantified as described in the legend to Fig. 3 (closed circles, solid line). This method quantifies the mass of the exogenous (14C-fatty acid). NEFA were also quantified by first fractionating total lipids on aminopropyl columns followed by RP-HPLC (closed squares, dashed line). The results are expressed as fatty acid mass (nmol/mg protein) and are representative of two separate studies. The mass levels of NEFA prior to fatty acid treatment are shown in Fig. 2. C, effect of 18:1n-9 and 20:5n-3 treatment on total unsaturated fatty acids in the NEFA fraction of primary hepatocytes. This graph illustrates changes in 18:1n-9 (white bars), 20:5n-3 (black bars), and other unsaturated fatty acids (UFA, gray bars) following treatment of cells with 18:1n-9 or 20:5n-3. The unsaturated fatty acids include 18:2n-6, 18:3n-3, 18:3n-6, 20:4n-6, 22:4n-6, and 22:6n-3. Veh, vehicle.

**Fig. 5.** Time course of 18:1n-9 and 20:5n-3 effects on hepatocyte mRNA encoding CYP4A. Primary hepatocytes were prepared and treated with fatty acids at 250 μM as described in the legend to Fig. 1. The cells were harvested at the times indicated to measure changes in mRNA (means ± S.D.). The study is representative of two separate studies with triplicate samples per group. Vehicle- and 18:1n-9-treated cells yielded similar results. 18:1n-9-treated cells (closed circles, solid line) and 20:5n-3-treated cells (closed squares, dashed line) are shown.
marginal effect on mRNA_{CYP4A} in primary hepatocytes (15, 16) and PPARα activity (Fig. 1). The combined treatment of 20:4n-6 and DDC induced CYP4A mRNA ~50% (Fig. 6). Treatment with 20:5n-3 alone induced the mRNA_{CYP4A} ~2.5; with DDC added, CYP4A mRNA increased nearly 4-fold. Similar effects were seen with 1-aminobenzoic acid, another inhibitor of microsomal oxidation (not shown). Thus, interference with microsomal fatty acid metabolism is sufficient to impact levels of this PPARα regulated transcript. These studies suggest that the generation of eicosanoids is not required for the 20:5n-3-mediated activation of PPARα in primary hepatocytes. In addition, these results also indicate that microsomal 20:4n-6 and 20:5n-3 metabolism may be important for degrading PPARα ligands in liver.

20-Carbon, but Not 18-Carbon, Unsaturated Fatty Acids Are Elongated in Cultured Primary Hepatocytes—In addition to the assimilation of exogenous fatty acids into complex lipids and oxidation, fatty acyl CoA thioesters serve as substrates for malonyl CoA-dependent elongation and NADPH-dependent desaturation. These modified fatty acids then serve as substrates for eicosanoid synthesis and peroxisomal ω-oxidation in mitochondria and peroxisomes. To assess these transformations, saponified lipids from the 14C-fatty acid labeling studies were fractionated by RP-HPLC (Fig. 7). As 14C-18:1n-9 accumulates in cells, <3% of 14C-18:1n-9 is elongated to a 20-carbon fatty acid. 18:2n-6 and 18:3n-3 were also poorly elongated to 20-carbon fatty acids in primary hepatocytes (not shown). Thus, >97% of the 18-carbon fatty acid supplied to hepatocytes enters complex lipids as the 18-carbon fatty acid.

In contrast, 14C-20:5n-3 is elongated to 22:5n-3 in primary hepatocytes over a 24-h period (Fig. 7B). The fraction accumulating as 22:5n-3 increases linearly with time, reaching ~30% of the total 14C-fatty acid in cells by 24 h. Similar studies with 20:4n-6 revealed ~25% of its conversion to 22:4n-6 (not shown). Some studies have indicated that 22:5n-3 is elongated to 24:5n-3, but its appearance is transient, reflecting ω-oxidation. However, no evidence of desaturation of any 18- or 20-carbon fatty acid was detected in these metabolic labeling studies. Thus, 14C-22:6n-3 is not generated in primary hepatocytes treated with 14C-18:3n-3 (not shown) or 14C-20:5n-3 (Fig. 7B). Consistent with this observation is the lack of increase in total cellular 22:6n-3 mass following 20:5n-3 administration to primary hepatocytes. The failure to generate 22:6n-3 from 18- and 20-carbon precursors is likely due to a decline in Δ9 and Δ11-desaturase activity when liver is explanted for primary hepatocyte culture. The reason for this decline is unknown.
thioesterase activity converts this inactive ligand to an active ligand (Fig. 9A).

**DISCUSSION**

We have examined the role hepatic metabolism plays in the control of cellular levels of certain natural ligands for PPARα. Although previous studies have suggested that oxidized lipids are prospective ligands for PPARα and PPARγ (eicosanoids) (2, 7–9), such metabolic routes do not apply to hepatic parenchymal cells because of the absence of robust cyclooxygenase or lipoxygenase activity in these cells (10). In fact, our studies argue against a requirement for the generation of oxidized lipids, *i.e.* eicosanoids, to activate PPARα in hepatocytes (Fig. 6). Instead, the presence of certain 20- and 22-carbon PUFA in the intracellular NEFA pool appear to represent major determinants controlling PPARα activity. The new information reported here includes: 1) the rapidity and magnitude of change in NEFA pool composition following fatty acid challenge; 2) dynamic changes in PPARα activity and mRNA_{CYP4A} abundance following the treatment of primary hepatocytes with 18:2, 20-, and 22-carbon PUFA; and 3) the identification of several biochemical reactions likely to be important for regulating cellular levels of PPAR ligands, *i.e.* microsomal fatty acid oxidation (Fig. 6) and elongation (Fig. 7), and peroxisomal β-oxidation and fatty acyl CoA thioesterase activity (Figs. 8 and 9). Together, these findings provide a biochemical basis for understanding the differential effects of 18:1n-9, 20:5n-3, 22:5n-3, and 22:6n-3 on PPARα activity in primary rat hepatocytes.

The relative abundance of several prospective nonesterified ligands for PPARα in primary hepatocytes is 18:1n-9 = 18:2n-6 = 20:4n-6 > 22:6n-3 > 18:3n-3 = 20:5n-3 (Fig. 2). Challenging cells with exogenous 18:1n-9, 18:2n-6, or 20:4n-6 has little effect on the mass of these fatty acids in the NEFA pool, PPARα activity, PPAR/retinoid X receptor activity, or PPAR-regulated transcripts, *e.g.* mRNA_{AOX} or mRNA_{CYP4A} (Figs. 1 and 4) (15, 16). In contrast, 20:5n-3 and 22:5n-3 are very low abundance fatty acids in primary hepatocytes (Fig. 2) and liver (22, 23), whereas 22:6n-3 is of intermediate abundance. The addition of 20:5n-3, 22:5n-3, or 22:6n-3 to hepatocytes significantly alters NEFA pool composition, but only 20:5n-3 and 22:6n-3 activate PPARα (Figs. 8 and 9). This suggests that major changes in NEFA pool composition/mass alone are insufficient to trigger a PPARα response; fatty acid structure is likely a key factor in this response.

The liver plays a major role in the elongation and desaturation of dietary 18:3n-3 and 20:5n-3 to 22:6n-3 (29). The metabolic pathway for the conversion of 20:5n-3 to 22:6n-3 is illustrated in Fig. 9A. The conversion of 18:3n-3 to 20:5n-3 requires both Δ5 and Δ6 desaturase and an elongase. Although primary hepatocytes retain the capacity to elongate 20-carbon PUFA and to β-oxidize 22-carbon PUFA (Figs. 7 and 9), they lose the
capacity for both $\Delta^8$ (not shown) and $\Delta^6$ desaturase activity (Fig. 7). The molecular basis for this loss is not known. If our cells retained an intact $\Delta^6$ desaturase system, the addition of 20:5-n-3 would lead to the synthesis of 22:6-n-3 at the expense of 22:5-n-3, and we would be unable to distinguish between the effects of 20:5-n-3, 22:5-n-3, and 22:6-n-3 on PPAR$\alpha$ activity. Because the presence of 22:5-n-3 has a marginal effect on 20:5-3 activation of PPAR$\alpha$, 22:5-n-3 appears to be inactive toward PPAR$\alpha$ (Fig. 8). Thus, elongating 20:5-n-3 to 22:5-n-3 represents a route for inactivation of a prospective PPAR$\alpha$ ligand.

The composition of the NEFA pool is influenced not only by exogenous fatty acids but also by endogenous metabolic events. Finding that the addition of either 22:5-n-3 or 22:6-n-3 to primary hepatocytes leads to the accumulation of 20:5-n-3 in the NEFA pool implicates a role for two peroxisomal functions. 22- and 24-carbon PUFA are preferentially $\beta$-oxidized in the peroxisome resulting in a reduction in chain length by 2 or 4 carbons (29). The resulting fatty acyl CoA thioesters must be hydrolyzed by a thioesterase for 20:5-n-3 to appear in the NEFA pool (Fig. 9). Previous studies with acyl CoA oxidase null mice suggested that the peroxisome was important for regulating PPAR$\alpha$ ligands (30). Our studies extend this observation to include the peroxisome as a key organelle for controlling cellular levels of 20- and 22-carbon PUFA ligands for PPAR$\alpha$.

Increased abundance of 20:5-n-3 and 22:6-n-3 in the NEFA pool correlates well with PPAR$\alpha$ activation in primary hepatocytes. Whether this same mechanism accounts for the dietary fatty acid regulation of PPAR$\alpha$ in hepatic and nonhepatic tissues in vivo remains unresolved. Some insight into the NEFA pool composition can be obtained by analysis of fatty acids co-isolated with FABP. Native L-FABP isolated from the livers of rats maintained on standard lab chow were found to contain endogenous fatty acids of various chain lengths, i.e. C$_{16-22}$: PUFA represented 44% of the total. Although L-FABP binds 20:5-n-3, 22:5-n-3, and 22:6-n-3 with affinities ranging from $\sim$50 to 250 nM (31), only 22:6-n-3 was associated with L-FABP at 1.9 mol % (24). The low abundance of 20:5-n-3 and 22:5-n-3 relative to 22:6-n-3 in liver probably accounts for this distribution of L-FABP-associated fatty acids (22, 23) (Fig. 2). Interestingly, the relative distribution of L-FABP-bound fatty acids reported by Murphy et al. (24), is remarkably similar to the composition of the NEFA pool derived from male rats maintained on a TekLad chow diet (Fig. 2). Nevertheless, feeding humans or rats fish oil, a rich source of n3-PUFA, is reported to increase hepatic CYP4A and acyl CoA oxidase induction (16), we predict that future studies will describe the enrichment of the NEFA pool and L-FABP with 20- and 22-carbon PUFA following fish oil feeding.

In conclusion, we have used PPAR$\alpha$ in rat primary hepatocytes as a model system to evaluate the role metabolism plays in the control of transcription factor activity. Our studies support the concept that dynamic changes in NEFA pool composition trigger PPAR$\alpha$ activation and induce PPAR$\alpha$-regulated gene transcription. Several key biochemical reactions appear to be important for controlling the hepatocyte levels of PPAR$\alpha$ ligands, including acyl CoA synthetase and thioesterase activities, fatty acid elongation, and desaturase activities as well as peroxisomal $\beta$-oxidation. Other nuclear receptors have been implicated as targets for fatty acid control, including hepatic nuclear factor-4 ($\alpha$ and $\gamma$), liver X receptor $\alpha$, retinoid-related orphan receptor, and retinoid X receptor (20, 33–36). The mechanisms described here may be important for controlling cellular levels of ligands regulating these other nuclear receptors.

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REFERENCES

1. Xu, H. E., Lambert, M. H., Montana, V. G., Parks, D. J., Blanchard, S. G., Brown, P. J., Sternbach, D. D., Lehmann, J. M., Wisely, G. B., Wilson, T. M., Kiewer, S. A., and Milburn, M. V. (1999) Mol. Cell. Biol. 19, 403–409
2. Devergine, B. and Wahl, W. (1999) Endocr. Res. 20, 649–688
3. McArthur, M. J., Atshaves, B. P., Frolov, A., Foxworth, W. D., Kier, A. B., and Schroeder, F. (1999) J. Lipid Res. 40, 1371–1383
4. Stahl, A., Gimeno, RE, Tartaglia, L., and Lodish, H. F. (2001) Trends Endocrinol. Metab. 12, 266–331
5. Lawrence, J. W., Kroll, D. J., and Racho, P. I. (2000) J. Lipid Res. 41, 1390–1401
6. Coleman, R. A., Lewin, T. M., and Muoio, D. M. (2000) Annu. Rev. Nutr. 20, 77–103
7. Forman, B. M., Chen, J., and Evans, R. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4312–4317
8. Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Ravera, C. P., McMahon, G., Brown, M., and Lazar, M. A. (1995) J. Biol. Chem. 270, 23975–23983
9. Kliewer, S. A., Sandseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4318–4323
10. Jump, D. B. (2002) J. Biol. Chem. 277, 8755–8758
11. Malkowski, M. G., Thuresson, E. D., Lakkides, K. M., Rieke, C. J., Micielli, R., Smith, W. L., and Garavito, R. M. (2001) J. Biol. Chem. 276, 37547–37555
12. Seo, T., Oelkers, P. M., Giattina, M. R., Worgall, T. S., Sturley, S. L., and Deckelbaum, R. U. (2001) Biochemistry 40, 4756–4762
13. Berge, R. K., Madsen, L., Vaagenes, H., Tronstad, K. J., Gottlicher, M., and Rustan, A. C. (1999) Biochem. J. 343, 191–197
14. Mater, M. K., Thelen, A. P., and Jump, D. B. (1999) J. Lipid Res. 40, 1045–1052
15. Ren, B., Thelen, A. P., and Jump, D. B. (1996) J. Biol. Chem. 271, 17167–17173
16. Ren, B., Thelen, A. P., Peters, J. M., Gonzalez, F. J., and Jump, D. B. (1997) J. Biol. Chem. 272, 26827–26832
17. Mater, M. K., Thelen, A. P., Pan, D. A., and Jump, D. B. (1999) J. Biol. Chem. 274, 32725–32732
18. Jump, D. B., Thelen, A. P., and Mater, M. K. (2001) J. Biol. Chem. 276, 34419–34427
19. Pan, D. A., Mater, M. K., Thelen, A. P., Peters, J. M., Gonzalez, F. J., and Jump, D. B. (2000) J. Lipid Res. 41, 742–751
20. Pawar, A., Xu, J., Jerks, E., Mangelsdorf, D. J., and Jump, D. B. (2002) J. Biol. Chem. 277, 39243–39250
21. Mata de Urquiza, A., Liu, S., Sjoberg, M., Zetterstrom, R. H., Griffith, W., Sjovall, J., and Perlmann, T. (2000) Science 290, 2140–2144
22. Salem, H. J. (1989) in Current Topics in Nutrition and Disease (Spiller, G. S., and Scala, J., eds) Vol. 22, pp. 109–128, Alan R. Liss, Inc., New York
23. Christiansen, E. N., Lund, J. S., Rortveit, T., and Rustan, A. C. (1991) Biochim. Biophys. Acta 1082, 57–62
24. Murphy, E. J., Edmondson, R. D., Russell, D. H., Colles, S and Schroeder, F. (1999) Biochim. Biophys. Acta. 1436, 413–425
25. Fisher, E.A., Pan, M., Chen, X., Wu, X., Wang, H., Jamil, H., Sparks, J. D., and Williams, K. J. (2001) J. Biol. Chem. 276, 27855–27863
26. Lang, C. A., and Davis, R. A. (1990) J. Lipid Res. 31, 2079–2086
27. Amet, Y., Berthou, F., Baird, S., Dreano, Y., and Menez, J. F. (1995) Biochem. Pharmacol. 50, 1775–1782
28. Luthria, D. L., Mohammed, B. S., and Sprecher, H. (1996) J. Biol. Chem. 271, 16020–16025
29. Sprecher, H. (2000) Biochim. Biophys. Acta 1486, 219–231
30. Pan, C. Y., Pan, J., Usuda, N., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. (1998) J. Biol. Chem. 273, 15629–15645
31. Norris, A. W., and Spector, A. A. (2002) J. Lipid Res. 43, 646–653
32. Liebich, H. M., Wirth, C., and Jakober, B. (1991) J. Chromatogr. 572, 1–9
33. Willson, T. M., and Moore, J. T. (2002) Mol. Endocrinol. 16, 1135–1144
34. Wisely, G. B., Miller, A. B., Davis, R. G., Thorquest, A. D., Jr., Johnson, R., Spitzer, T., Sefer, A., Shearer, B., Moore, J. T., Willson, T. M., and Williams, S. P. (2002) Structure 10, 1225–1234
35. Dhe-Paganon, S., Duda, K., Iwamoto, M., Chi, Y. I., and Shoelson, S. E. (2002) J. Biol. Chem. 277, 37973–37976
36. Ou, J., Tu, H., Shan, B., Lu, A., DeBose-Boyd, R. A., Bashmakov, Y., Goldstein, J. L., and Brown, M. S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6027–6032
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