Cytochrome P450 Eicosanoids are Activators of Peroxisome Proliferator-Activated Receptor α

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Received November 10, 2006; accepted April 9, 2007

ABSTRACT:

Cytochrome P450 (P450) eicosanoids regulate vascular tone, renal tubular transport, cellular proliferation, and inflammation. Both the CYP4A ω-hydroxylases, which catalyze 20-hydroxyeicosatetraenoic acid (20-HETE) formation, and soluble epoxide hydrolase (sEH), which catalyzes epoxide-hydroxyeicosatrienoic acid (EET) degradation to the dihydroxyeicosatrienoic acids (DHETs), are induced upon activation of peroxisome proliferator-activated receptor α (PPARα) by fatty acids and fibrates. In contrast, the CYP2C epoxygenases, which are responsible for EET formation, are repressed after fibrate treatment. We show here that P450 eicosanoids can bind to and activate PPARα and result in the modulation of PPARα target gene expression. In transactivation assays, 14,15-DHET, 11,2-EET, and 20-HETE were potent activators of PPARα. Gel shift assays showed that EETs, DHETs, and 20-HETE induced PPARα-specific binding to its cognate response element. Expression of apolipoprotein A-I was decreased 70% by 20-HETE, whereas apolipoprotein A-II expression was increased up to 3-fold by 11,12-EET, 14,15-DHET, and 20-HETE. In addition, P450 eicosanoids induced CYP4A1, sEH, and CYP2C11 expression, suggesting that they can regulate their own levels. Given that P450 eicosanoids have multiple cardiovascular effects, pharmacological modulation of their formation and/or degradation may yield therapeutic benefits.

Eicosanoids generated from arachidonic acid metabolism by cytochrome P450 (P450) enzymes are important autocrine and paracrine factors that have diverse biological functions. P450 eicosanoids are involved in the regulation of vascular tone, renal tubular transport, cardiac contractility, cellular proliferation, and inflammation (Roman, 2002). The major products of P450-catalyzed arachidonic acid metabolism are 19- and 20-hydroxyeicosatetraenoic acid (19- and 20-HETE), and the regio- and stereoisomeric epoxyeicosatrienoic acids (EETs) (Roman, 2002). 20-HETE formation is catalyzed by the CYP4A (Nguyen et al., 1999; Wang et al., 1999) and CYP4F (Powell et al., 1998; Xu et al., 2004) family of enzymes, whereas the EETs are products of arachidonic acid metabolism by the CYP2C and CYP2J enzymes (Karara et al., 1993; Wu et al., 1996, 1997). EETs are subsequently metabolized by the soluble epoxide hydrolase (sEH) into dihydroxyicosatrienoic acids (DHETs) (Zeldin et al., 1993; Yu et al., 2000).

Regulation of P450 eicosanoid levels is determined by many factors, including the induction or repression of the P450 enzymes responsible for their formation. Large numbers of studies have focused on the induction of rat CYP4A protein and mRNA levels by antihyperlipidemic agents such as clofibrate (Kimura et al., 1989). Rat renal CYP2C23 has also been shown to be inducible by fibrates (Muller et al., 2004), whereas rat hepatic CYP2C11 and CYP2C12, but not CYP2C13, are repressed (Corton et al., 1998). Hepatic and renal sEH expression in rodents is also induced after treatment with clofibrate (Pinot et al., 1995).

Fibrate drugs are part of a diverse group of compounds known as peroxisome proliferators, which also include herbicides and phthalate ester plasticizers (Bishop-Bailey, 2000). Peroxisome proliferators act via peroxisome proliferator-activated receptor α (PPARα) (Issemann and Green, 1990). This receptor is a member of the PPAR nuclear receptor family that also consists of the PPARγ and PPARδ isoforms. PPARα is mainly expressed in the heart, liver, and kidney, whereas the expression of PPARγ is predominantly in the adipose tissue (Bishop-Bailey, 2000). The biological role of PPARα as a lipid sensor...
has been well established. In primary rat hepatocytes, PPARα activation results in the up-regulation of apolipoprotein A-I (apoA-I) and apoA-II and increased expression of β-oxidation enzymes, fatty acid transport proteins, fatty acid binding proteins, and other genes involved in the control of triglyceride and fatty acid metabolism (Fruholt et al., 2001). In human hepatocytes, however, apoA-I expression is decreased after PPARα activation (Vu-Duc et al., 1998). The species-specific differences in response to PPARα agonists have been attributed to the expression level of PPARα (Palmer et al., 1998), which is significantly lower in humans than in rodents, as well as differences in the regulatory regions of the responsive genes (Vu-Duc et al., 1998; Cheema and Agellon, 2000).

More recently, both PPARα and PPARγ have also been found in vascular smooth muscle and endothelial cells (Bishop-Bailey, 2000). In both smooth muscle and endothelial cells derived from human and bovine sources, PPARα inhibits the inflammatory response by repressing NF-κB signaling. Expression of genes involved in inflammation, such as interleukin-6, cyclooxygenase 2, and vascular adhesion molecule, is inhibited after PPARα activation (Bishop-Bailey, 2000).

Mechanistically, PPARs are ligand-activated transcription factors that, upon ligand binding, will heterodimerize with the retinoic X receptor and bind to its response element, the peroxisome proliferator response element (PPRE). Ligands for PPARα include fatty acids, eicosanoids, and fibrate drugs (Forman et al., 1997). One of the more potent endogenous activators has been identified as the eicosanoid 8(S)-HETE (Forman et al., 1997). 8(S)-HETE is generated from the lipoxygenase pathway of arachidonic acid metabolism and activates and binds to PPARα at nanomolar concentrations. Other related eicosanoids such as prostacyclin, some prostaglandins, and leukotriene B4 also activate PPARα (Devchand et al., 1996). A more recent report also showed that ω-hydroxylated EETs are high-affinity ligands of PPARα (Cowart et al., 2002).

Given that related eicosanoids bind to and activate PPARα, and that P450s and sEH are regulated by peroxisome proliferators, we hypothesize that P450 eicosanoids are also agonists of PPARα. To test whether P450 eicosanoids can functionally activate PPARα, transactivation assays were carried out and demonstrated that 11,12-EET, 14,15-DHET, and 20-HETE are potent activators of PPARα and PPARγ. By using gel shift assays, P450 eicosanoids were shown to induce the binding of PPARs to a PPRE. Furthermore, we found that 11,12-EET and 14,15-DHET behave like peroxisome proliferators in that they are able to alter apoA-I and apoA-II mRNA expression in primary rat hepatocytes. Because apoA-I and apoA-II are involved in the transport of high-density lipoprotein, these findings suggest that P450 eicosanoids may play a role in the regulation of triglyceride levels via PPARα. In addition, we show that P450 and sEH mRNA levels were increased in primary rat hepatocytes after treatment with these eicosanoids. These results suggest that P450 eicosanoids may regulate their own levels through a complex autoregulatory mechanism.

Materials and Methods

Materials. Wy-14,643 and ciglitazone were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA) and eicosanoids were purchased from Cayman Chemical (Ann Arbor, MI). The EET agonist 11,12-epoxyeicosanoic acid (11,12-EZEE) was synthesized as described previously (Falk et al., 2003). 3,5,5-Triiodo-L-thyronine (T3) was purchased from Sigma Chemical Co. (St. Louis, MO). CYP4A1 and CYP2C11 primary antibodies were purchased from BD Gentest (Woburn, MA). The sEH antibody was a kind gift from Dr. Bruce Hammad (University of California, Davis, CA), and the CYP2C23 antibody was a kind gift from Dr. Jorge Capdevila (Vanderbilt University, Nashville, TN). Gal4 expression and reporter plasmids were provided by Dr. Thomas Scanlan (University of California, San Francisco, CA) and pCMV-mPPARα and pRS-hRXRe2 plasmids were a generous gift from Dr. Ronald Evans (Salk Institute, La Jolla, CA).

Cell Culture. HepG2 cells were obtained from The American Type Culture Collection (Manassas, VA) and maintained in modified Eagle’s medium with Earle’s balanced salt solution and contained 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and penicillin-streptomycin. Sprague-Dawley primary hepatocytes were isolated by the University of California, San Francisco Liver Center Core Facility, were cultured on collagen type I plates (BD Biosciences, Bedford, MA) and overlaid with 0.25 mg/ml Matrigel (BD Biosciences) in Hepocyte Culture Medium (Cambrex Bio Science Walkersville, Inc., Walkersville, MD). Primary hepatocytes were treated on the third day after isolation. All cells were cultured in 37°C with 5% CO2.

Transactivation Assays. HepG2 cells were plated in 24- well plates at 8 × 104 cells per well and transfected the next day using Lipofectamine PLUS reagents (Invitrogen, Carlsbad, CA) with 0.1 to 1 ng of Gal4-hPPARα or Gal4-hPPARγ, 100 ng of UASγ-TLUC, and 35 ng of pCMV-βgal. After 24 h, cells were dosed with 50 μM Wy-14,643, 10 μM ciglitazone, or 10 μM P450 eicosanoids in serum-free medium containing up to 0.1% DMSO for 6 h. Cells were lysed using Reporter Lysis Buffer (Promega, Madison, WI) and subjected to one freeze-thaw cycle. A single-tube format luminometer (MGM Instruments, Hamden, CT) was used for obtaining luciferase values using the Luciferase Assay Reagent as substrate (Promega). To determine β-galactosidase activity, cell lysates were incubated with the substrate 2-nitrophenyl-β-D-galactopyranoside and absorbance was measured at 420 nm. Luciferase activity is expressed relative to β-galactosidase activity, and -fold activation is calculated relative to DMSO control. Each experiment was carried out in quadruplicate and repeated three times.

Lipid Extraction and Liquid Chromatography-Tandem Mass Spectrometry. A confluent culture of HepG2 cells in a T75 flask was incubated with 10 μM P450 eicosanoids for 6 h. Extraction of lipids from culture medium and cells has been described previously (Fang et al., 2001). In brief, lipids from cell culture medium were extracted twice with water-saturated ice-cold ethyl acetate in a ratio of 1:4 (v/v). For the extraction of intracellular lipids, cells were first trypsinized and washed with phosphate-buffered saline, then homogenized by hand with a Dounce homogenizer. Ice-cold chloroform/methanol (2:1 v/v) was then added and the organic phase was removed. In some cases, hydrolysis of intracellular lipids was carried out by adding methanol containing 5% NaOH and 10% H2O to the organic phase and incubating the reaction for 1 h at 50°C. Lipids were then extracted with ethyl acetate as described above. The extracted layer was evaporated under nitrogen gas and stored at ~80°C. On the day of analysis, lipids were reconstituted in acetonitrile (100 μl). Quantitation of eicosanoids has been described previously (Yu et al., 2004). In brief, analysis of lipids by high-performance liquid chromatography was performed using a 4.6 × 150 mm, 5-μm Luna C18(2) column (Phenomenex, Torrance, CA) and lipids were eluted from the reverse-phase high-performance liquid chromatography column. The effluent was then injected into a Quattro Ultima tandem-quadrupole mass spectrometer (Waters, Milford, MA) and subjected to negative mode electrospray ionization. Multi-reaction monitoring was used to detect the eicosanoids simultaneously, and data were analyzed using the MassLynx 3.5 software (Waters).

Gel Shift Assays. pCMX-mPPARα (1 μg) and pRS-hRXRe2 (1 μg) were translated using the TNT reticulocyte in vitro translation system (Promega). The binding reaction contained 1 μl of translated PPARα, 0.3 μl of RXRe2 in 10 mM Tris, 150 mM KCl, 6% glycerol, 0.05% Igepal (Sigma), 1 mM dithiothreitol, 2 μg of poly(dI-dC), and P450 eicosanoids with or without 1 μl of PPARα or RXRe2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The reactions were incubated for 10 min on ice before 250,000 cpm of γ-32P-labeled PPRE was added. The sequence for the consensus PPRE oligonucleotide is 5′-AAAACTAGGTCAAAAGGTCA-3′, and the sequence for the mutant oligonucleotide is 5′-AAAAAGTAGACCAAGCACGA-3′. The oligonucleotides were end-labeled with γ-32P using T4 polynucleotide kinase. After incubation for 30 min at room temperature, the reaction was separated on a 5% pre-run polyacrylamide gel at 4°C. The gel was dried and radioactive bands were visualized using a PhosphorImager and ImageQuant software (GE Healthcare Biosciences, Chalfont St. Giles, Buckinghamshire, UK).

Real-Time Quantitative PCR. Primary hepatocytes were treated with
peroxisome proliferators or eicosanoids for 24 to 48 h. RNA was isolated using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was carried out using M-MLV reverse transcriptase (Promega). Primers and probe sets were designed using Primer Express. Results

### P450 Eicosanoids Transactivate PPARs

Transactivation assays were carried out to determine whether EETs or DHETs were able to functionally activate PPARα and PPARγ (Fig. 1). HepG2 cells were transfected with a Gal4 plasmid containing the ligand-binding domain of either human PPARα or PPARγ (Gal4-PPARα or Gal4-PPARγ) and a luciferase reporter plasmid containing four repeats of the Gal4 response element (UAS4-LUC). In cells not transfected with PPARα or PPARγ, only minimal basal activation was observed. When PPARα- or PPARγ-transfected cells were treated with the PPARα-specific activator Wy-14,643, hPPARα was activated greater than 30-fold. Similarly, hPPARγ was transactivated by ciglitazone, a PPARγ ligand. Significant activation was observed when hPPAR-transfected cells were dosed with 10 μM 11,12-EET, 11,12-EEZE, or 14,15-EET (Fig. 1A). The highest activation was observed with 11,12-EEZE, a monounsaturated 11,12-EET analog. 8,9-EET did not significantly activate either PPARα or PPARγ.

The effects of DHETs on hPPAR activation were also examined (Fig. 1B). hPPAR-transfected HepG2 cells were treated with 10 μM 8,9-DHET, 11,12-DHET, or 14,15-DHET. 14,15-DHET was the most potent activator and was as effective as Wy-14,643 and ciglitazone at transactivating the respective PPARs. hPPARα was activated 30-fold and hPPARγ 21-fold by 14,15-DHET. Activation of hPPARα and hPPARγ by 11,12-DHET was 8- to 9-fold. 8,9-DHET did not significantly activate either receptor. 20-HETE activated hPPARα 18-fold and hPPARγ 23-fold (Fig. 1C). To exclude the possibility that the activation observed with P450 eicosanoids was nonspecific for nuclear receptors, we tested their ability to transactivate an unrelated receptor, the thyroid hormone receptor β (TRβ) (Fig. 1D). The endogenous ligand of TRβ, T3, strongly activated this receptor, whereas 11,12-EET and 14,15-DHET did not have any effect on TRβ activation.

14,15-DHET was further tested for its ability to transactivate

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**Table 1**

<table>
<thead>
<tr>
<th>Primer or Probe</th>
<th>Sequence</th>
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<tr>
<td>Rat cyclophilin</td>
<td>5′-CCATGACGAGCCTCTGGA-3′</td>
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<tr>
<td>Reverse Primer</td>
<td>5′-TGTCGCTTGGTGGTGGCAGACTCTGTC-3′</td>
</tr>
<tr>
<td>Probe</td>
<td>6FAM-CCCTGCTGGTGGTGGCAGACTCTGTC-BHQ</td>
</tr>
<tr>
<td>Rat CYP4A1</td>
<td>5′-CTCATCCCTCTCCACCTGTT-3′</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5′-TCCACACATTCTCATATTCTCCTGAT-3′</td>
</tr>
<tr>
<td>Probe</td>
<td>6FAM-TCCACACATTCTCATATTCTCCTGAT-BHQ</td>
</tr>
<tr>
<td>Rat sEH</td>
<td>5′-CTCTAATCGTATCATGAAACAGAGAAG-3′</td>
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<tr>
<td>Reverse Primer</td>
<td>5′-CTCTAATCGTATCATGAAACAGAGAAG-3′</td>
</tr>
<tr>
<td>Probe</td>
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<tr>
<td>Rat CYP2C11</td>
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<td>Reverse Primer</td>
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<td>Probe</td>
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<tr>
<td>Rat CYP2C23</td>
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</tr>
<tr>
<td>Reverse Primer</td>
<td>5′-CCTCCAAACACACACCGCTCAGAT-5′</td>
</tr>
<tr>
<td>Probe</td>
<td>6FAM-AGAGTGCACAGACGAAGTATCAGAGGA-BHQ</td>
</tr>
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**Statistics.** Statistical significance of differences between values was evaluated by an unpaired Student’s t test. Significance was set at a p value of <0.05.

**Fig. 1.** P450 eicosanoids transactivate hPPARα and hPPARγ in HepG2 cells. Transactivation assays were performed in HepG2 cells that were transfected with Gal4-hPPARα or Gal4-hPPARγ and the reporter UAS4-LUC. Wy-14,643 (Wy, 50 μM), ciglitazone (Cig, 10 μM), or EETs (10 μM) (A), DHETs (10 μM) (B), or 20-HETE (10 μM) (C) were added to cells in serum-free medium 24 h post-transfection and cells were lysed 6 h later. D, HepG2 cells were transfected with Gal4-TRβ and UAS4-LUC and treated with 10 μM 11,12-EET and 10 μM 14,15-DHET as described above. Luciferase activity was normalized to β-galactosidase values and fold activation was calculated as increase over the DMSO control (transfected only with UAS4-LUC). The values shown are the mean ± S.D. of a representative experiment performed in quadruplicate. Similar results were obtained in additional experiments. * significant difference compared to DMSO (p < 0.05).
Transactivation assays were performed as described in Fig. 1. Transactivation of a dose-dependent decrease in mPPAR of cold PPRE were incubated with the complex, which resulted in B–E). To further validate binding specificity, increasing amounts of a representative experiment performed in quadruplicate. EC50 values of 1.5 spectrometry (Table 2). In cells treated with 10

Characterization of the Distribution of EETs and DHETs in HepG2 Cells. To characterize the availability of eicosanoids in our cellular system, the distribution of exogenously administered P450 eicosanoids was examined using liquid chromatography-tandem mass spectrometry (Table 2). In cells treated with 10 μM 11,12-EET, approximately 48% was metabolized into 11,12-DHET. Of the 52% 11,12-EET remaining, 32% was detected in the extracellular medium, and 20% was incorporated into phospholipid pools; unbound intracellular 11,12-EET was not detected. The 11,12-DHET formed from 11,12-EET was largely detected in the extracellular medium (47%), with 0.7% found in phospholipid pools and 0.2% as free intracellular lipids. Treatment with 10 μM 14,15-DHET resulted in the predominant distribution of 14,15-DHET extracellularly (99%), with a small percentage bound within lipid pools.

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EETs and DHETs Induce PPAR/RXR Binding to a PPRE. Gel shift assays were carried out to determine whether P450 eicosanoids could induce a conformational change in PPARα, resulting in subsequent binding of the PPARα/RXRα heterodimer to a PPRE. EETs, DHETs, and 20-HETE induced heterodimer binding, which was not observed with a mutant PPRE or unprogrammed reticulocytes (Fig. 3A). Supershifts with mPPARα and RXRα specific antibodies demonstrated that heterodimers consisted of mPPARα and RXRα (Fig. 3, B–E). To further validate binding specificity, increasing amounts of cold PPRE were incubated with the complex, which resulted in a dose-dependent decrease in mPPARα/RXRα bound to radiolabeled PPRE (Fig. 3F). Likewise, 20-HETE induced mPPARα/ RXRα-specific heterodimerization and subsequent binding to the PPRE (Fig. 3G).

P450 Eicosanoids Alter the Expression of PPARα-Responsive Genes. After demonstration of PPARα activation by P450 eicosanoids, it was of interest to investigate whether P450 eicosanoids were able to mimic the actions of peroxisome proliferators and alter the RNA levels of known PPARα-responsive genes involved in the metabolism and transport of triglycerides. Primary hepatocytes from Sprague-Dawley rats were treated ex vivo with 10 μM 11,12-EET, 14,15-DHET, or 20-HETE, and RNA expression of apoA-I, apoA-II, and CPT1A was determined by real-time quantitative PCR (Fig. 4A). Wy-14,643 and 14,15-DHET slightly decreased apoA-I expression. Interestingly, 20-HETE resulted in a 70% decrease in apoA-I expression. In contrast, apoA-II expression was increased after treatment with PPARα activators. Treatment with Wy-14,643 resulted in a 2-fold increase in apoA-II expression, and induction was more than 3-fold with 11,12-EET, 14,15-DHET, and 20-HETE. The expression of CPT1A was only minimally affected by P450 eicosanoids, in contrast to potent activation by Wy-14,643.

Because peroxisome proliferators can modulate P450 and sEH levels, we investigated whether P450 eicosanoids had the ability to modify P450 and sEH levels, thereby regulating their own expression (Fig. 4B). As expected, Wy-14,643 resulted in a marked increase in CYP4A1 and sEH expression. In contrast, CYP4A1 and sEH mRNA levels were increased only 2- to 3-fold by 10 μM 11,12-EET, 14,15-

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Extracellular</th>
<th>Free Intracellular</th>
<th>Lipid Bound Intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>11,12-EET Treatment</td>
<td>32.1 ± 13.5</td>
<td>N.D.</td>
<td>19.8 ± 2.97</td>
</tr>
<tr>
<td>11,12-DHET</td>
<td>47.3 ± 7.57</td>
<td>0.12 ± 0.013</td>
<td>0.69 ± 0.15</td>
</tr>
<tr>
<td>14,15-DHET Treatment</td>
<td>99.9 ± 34.0</td>
<td>0.0015 ± 0.0001</td>
<td>0.0145 ± 0.0045</td>
</tr>
</tbody>
</table>

* The amounts of 11,12-EET and 11,12-DHET formed from 11,12-EET are expressed as a percentage of the sum of these two eicosanoids.

* The amount of 14,15-DHET present in the extracellular medium or intracellularly following DHET dosing is expressed relative to the total 14,15-DHET content.

### Discussion

P450 eicosanoids are identified as novel PPARα and PPARγ activators. Of the eicosanoids tested, 11,12-EET, 14,15-DHET, and 20-HETE are the most potent activators of both isoforms. It is recognized that the exogenous concentrations added to culture medium are likely to be higher than intracellular eicosanoid levels. A significant attenuation of PPAR activation by P450 eicosanoids dosed in serum-containing medium is consistent with binding to cellular proteins and reduced free intracellular concentrations (data not shown). Of the exogenously administered 11,12-EET, no free fatty acids were detected intracellularly, and approximately 2 μM was bound in phospholipid pools. This concentration represents one-fifth of the initially administered 11,12-EET dose and suggests that the concentration of free 11,12-EET within the cell is even lower than 2 μM. In vivo,
FIG. 3. P450 eicosanoids induce mPPARα/RXRα-PPRE binding. Electrophoretic mobility shift assays were performed by incubating in vitro translated mPPARα and RXRα with DMSO control, 5 μM Wy-14,643, or 1 μM P450 eicosanoids and 32P-labeled PPRE. The complex was resolved through a 5% nondenaturing polyacrylamide gel and analyzed using a PhosphorImager. Mutant PPRE (Mut) and unprogrammed reticulocyte (Unpgr) were used as controls (A and B). Specificity of the EET (B and D), DHET (C and E), and 20-HETE (G) effects were determined by supershifts with a PPARα (B, C, and G) and RXRα (D, E, and G) antibody. N.S. denotes nonspecific binding. Competition assays were performed with increasing amounts of cold PPRE (F and G).
endogenous EET levels in rodent plasma (Z. Yu and D. L. Kroetz, unpublished results) and human liver (Karara et al., 1991) are in the nanomolar range. The ED_{50} values obtained from transactivation assays in this report were in the low micromolar range; consideration of the ratio of the EET dose to the level of lipid-bound EETs measured intracellularly (~5:1) would be consistent with intracellular eicosanoid concentrations in the nanomolar range, making it likely that 11,12-EET can activate PPARα and PPARγ in vivo.

The fact that 11,12-EET-derived DHET was detected predominantly in the extracellular medium suggests that, subsequent to metabolism by intracellular sEH, 11,12-DHET was secreted into the medium. Although almost all of the exogenously administered 14,15-DHET was found in the culture medium, this finding does not indicate that 14,15-DHET was not available intracellularly. It is likely that, akin to 11,12-DHET, 14,15-DHET is secreted after entry into the cell. The above phenomenon makes it difficult to interpret the free intracellular concentrations of DHETs that are available to activate PPARs in the cellular system used in these studies, but it is likely to be submicromolar.

Regioisomeric P450 eicosanoids may be preferentially metabolized in the cell, thus influencing PPAR activation. Differences in PPARα activation by regioisomeric EET/sEH/DHET pairs suggest that the DHET alone cannot account for all of the activity associated with EET treatment. To address the relative contributions of EETs and DHETs, it would be of interest to measure EET transactivation in a cell system devoid of sEH activity. Unfortunately, inhibition of sEH by ureabased and chalcone oxide sEH inhibitors was not possible in these studies since these inhibitors also activated PPARs (V. Y. Ng and D. L. Kroetz, unpublished data; Ng et al., 2006). Further studies would be required to adequately address the relative contribution of EETs and DHETs in the observed activation of PPAR.

11,12-EET and 14,15-DHET transactivated human PPARα and PPARγ to a similar extent. Many reports have shown the dual activation of PPARs by polyunsaturated fatty acids. Linoleic, arachidonic, and eicosapentaenoic acids activate murine PPARα and PPARγ (Forman et al., 1997), and palmitic, oleic, linolenic, and arachidonic acids are agonists for murine PPARα and PPARγ (Kliwer et al., 1997). In recent years, increased efforts to synthesize compounds that act as PPARα/PPARγ dual agonists such as ragaglitazar (Brand et al., 2003) and MK-0767 (Doebber et al., 2004) have shown that these compounds have beneficial effects on insulin resistance and display antihyperglycemic and hypolipidemic activities. Because P450 eicosanoids are endogenous compounds that are released into the intracellular environment, it is of interest to explore whether P450 eicosanoids play a role in improving insulin sensitivity and maintaining lipid homeostasis.

It has been shown that 10 to 20 μM ω-hydroxylated EETs transactivated Gal4-mPPARα about 2- to 3-fold over control (Cowart et al., 2002). In the present study, 10 μM 11,12-EET and 14,15-EET activated Gal4-hPPARγ more than 10-fold, suggesting that the non-ω-hydroxylated EETs may be more potent activators of PPARs. It is important, however, to take into account the differences in cell type and the species of PPARα that were used in the assays. 20,14,15-HEET displaced cis-parinaric acid from the ligand-binding domain of PPARα with high affinity (K_{i} = 3 nM). The corresponding K_{i} values for EETs and Wy-14,643 are about 10- and 26-fold higher, respectively (Cowart et al., 2002). On the basis of ligand displacement assays, EETs and Wy-14,643 are expected to have lower affinity for PPARα than have the hydroxylated EETs. However, Wy-14,643 was functionally as potent at transactivating full-length PPARα as 20,14,15-HEET, and in assays using Gal4-mPPARγ chimeras, Wy-14,643 was 4 times more potent than 20,14,15-HEET. The lack of correlation between cis-parinaric acid displacement constants and transactivation potential makes it difficult to assign relative potencies for PPARα activation to the P450 eicosanoids. The possibility exists that in vivo, EETs are converted to their hydroxylated products in the presence of the CYP4A enzymes. However, CYP4A expression was not detected in HepG2 cells, where the present transactivation studies were performed, consistent with a direct effect of EETs on PPARα.

8(5)-HETE, 15-deoxy-Δ^{12,14}-prostaglandin J_{2}, and hydroxylated eicosanoids are naturally occurring eicosanoid ligands for PPARα and PPARγ (Forman et al., 1997; Kliwer et al., 1997; Cowart et al., 2002). Given the similarity in structure between these established ligands and P450 eicosanoids, and the promiscuous ligand-binding pocket of PPARs, it is not surprising that P450 eicosanoids can also activate PPARs. A major determinant of substrate specificity between PPARα and PPARγ is the Tyr314 residue. It will be interesting to model whether the dual agonist P450 eicosanoids can efficiently hydrogen bond with Tyr314 and its equivalent in PPARγ. Preliminary docking efforts to examine this interaction were hindered by the large number of configurations that P450 eicosanoids can assume. Structural information will provide additional insight as to why certain P450 eicosanoids are better agonists than others despite their high degree of structural similarity.

Most of the interest in PPARα activation has focused on its role in regulating lipid homeostasis. The degree to which P450 eicosanoids decreased apoA-I mRNA levels in primary rat hepatocytes was similar to or greater than that of the well characterized PPARα agonist Wy-14,643. The regulation of the apoA-I gene by PPARα is specie-
and ligand-specific (Vu-Dac et al., 1998). In previous rat hepatocyte studies, fenofibrate transcriptionally decreased apoA-I mRNA, whereas benzbafibrate, gemfibrozil, and Wy-14,643 had no effect (Staels et al., 1992). The difference in Wy-14,643 effects between the studies could be due to differences in rat strain and the mode of activator exposure. Because P450 eicosanoids cannot be administered in vivo, hepatocytes were dosed after isolation. In contrast, for the fibrate studies, hepatocytes were isolated after in vivo administration of the activators (Staels et al., 1992). Remarkably, 20-HETE was significantly more potent and resulted in a 70% decrease in apoA-I mRNA. It may be possible that the 20-HETE effect is not due solely to PPARα activation. The RXR homodimer (Nagasaki et al., 1994), Rev-erβ (Vu-Dac et al., 1998), hepatic nuclear factor-4 (Chan et al., 1993), and saturated fatty acids (Srivastava, 1994) have been implicated in apoA-I regulation. It is important to note, however, that in contrast to rodents, PPARα activation in human hepatocytes leads to an increase in apoA-I expression (Berthou et al., 1996), which is regulated by more complex mechanisms. The effect of P450 eicosanoids on apoA-I expression in humans requires further study.

apoA-II expression in rat hepatocytes was significantly induced after treatment with Wy-14,643 and P450 eicosanoids. In earlier studies, fenofibrate decreased apoA-II mRNA in the rat liver (Staels et al., 1992) and increased hepatic production of apoA-II in humans (Vu-Dac et al., 1995). The conflicting effects of PPARα agonists on apoA-II expression may be related to strain-specific regulation of this gene, as has been previously reported for rat CYP4A (Sewer et al., 1996). In Fischer 344 rats, the lauric acid ω-hydroxylase activity was induced 1.6-fold, whereas ω-1 activity decreased 38% in response to lipopolysaccharide treatment. In Sprague-Dawley rats, however, both ω- and ω-1 activities decreased. Corresponding CYP4A protein levels were also not consistent between Fischer and Sprague-Dawley rats. In comparing the response of P450s to lipopolysaccharide stimulation between Fischer and Sprague-Dawley rats, the authors (Sewer et al., 1996) found that in general, the results obtained from Fischer rats were more consistent and reproducible than that from Sprague-Dawley rats. It is possible that regulatory regions of the CYP4A gene may be different between these strains, leading to differences in response to the same stimulus. As is the case with apoA-I, we cannot rule out the possibility that P450 eicosanoids mediate their effects via other mechanisms.

The ability of P450 eicosanoids to regulate their own levels via PPARα was examined by looking at their effects on PPARα-responsive genes in the arachidonic acid metabolism cascade (summarized in Fig. 5). mRNA expression of CYP4A and sEH is highly responsive to treatment with fibrates and Wy-14,643 (Kimura et al., 1989; Tollet et al., 1994; Pinot et al., 1995). In this study, moderate differences in CYP4A1 and sEH RNA expression were observed with P450 eicosanoid treatment. One possibility for the minimal effect of P450 eicosanoids on CYP4A1 and sEH in hepatocytes, despite potent transactivation of PPARα in vitro, is an opposing effect of PPARα and PPARγ activation on the expression of these genes. Although the role and expression of PPARγ has been mainly described in the adipose tissues and macrophages (Bishop-Bailey, 2000), PPARγ does exhibit weak expression in murine (Vidal-Puig et al., 1996) and human liver (Semple et al., 2006). Under pathological conditions such as hepatic steatosis and obesity, the hepatic expression of PPARα can also be up-regulated (Vidal-Puig et al., 1996; Gavrilova et al., 2003). It has been shown that the uncoupling protein 1 promoter can be responsive to both PPARα and PPARγ (Barbera et al., 2001). Troglitazone, an agonist of both PPARα and PPARγ, has opposing effects on PPARα and PPARγ expression in mononuclear cells (Aljada et al., 2001). The transrepressive effect of PPARγ in macrophages has also been described (Pascual et al., 2005; Ghisletti et al., 2007). Since Wy-14,643 is highly specific for PPARα, the expected induction of CPT1A, CYP4A1, and sEH was observed (Fig. 5), whereas when hepatocytes were treated with P450 eicosanoids, the potentially opposing dual effects elicited by both PPARα and PPARγ may have accounted for the moderate increase in the expression of these genes. It will be of interest in future studies to determine whether the dichotomous effects of Wy-14,643 and P450 eicosanoids on PPAR-responsive genes in rat hepatocytes are due to the dual activation of PPARα and PPARγ. Experiments in which the expression of each PPAR isoform is silenced could be used to isolate the effect of these two related nuclear receptors.

In contrast to CYP4A and sEH, CYP2C11 expression was markedly induced after treatment with P450 eicosanoids. Of note is that the P450 eicosanoids are more potent in inducing CYP2C11 than is Wy-14,643. It has previously been reported that dietary administration of Wy-14,643, gemfibrozil, and di-n-butyl phthalate suppresses hepatic CYP2C11 expression in Fischer 344 and Sprague-Dawley rats (Corton et al., 1998). A similar decrease in CYP2C11 protein levels was observed after treatment of Fisher 344 rat hepatocytes with Wy-14,643 (Corton et al., 1998). The contrasting effects of Wy-14,643 on CYP2C11 expression in the present study and in this earlier study could be due to differences in the administered dose. It is difficult to compare our results with the earlier in vivo studies, in which Wy-14,643 was administered on a ppm basis for up to 13 weeks. However, in the previous rat hepatocyte studies, higher concentrations of Wy-14,643 (100–400 μM) were used than in our study (50 μM) and CYP2C11 down-regulation was not apparent until a concentration of 200 μM. It is possible that at lower concentrations of Wy-14,643, activation of PPARα is observed, whereas at higher concentrations, repression is apparent. A similar concentration-dependent effect has been reported for the PPARα activator, dehydroepiandrosterone (Ripp et al., 2003). Another possibility is that differences in the hepatocyte culture conditions between the two studies influenced the effect of Wy-14,643 on CYP2C11 expression. In the present study, hepatocyte conditions were optimized with respect to CYP4A induction by Wy-14,643, and these conditions may support activation and not repression of CYP2C11 by PPARα activators. As with the response seen with apoA-I and apoA-II, the
route of agonist exposure may also affect the importance of the roles the agonist can elicit on gene expression.

One of the best characterized roles of PPARγ is the induction of adipocyte differentiation (Forman et al., 1995). Activation of PPARγ can lead to an increase in proteins involved in lipid storage and metabolism during adipogenesis, such as adipocyte fatty acid binding protein and phosphoenolpyruvate carboxykinase (Tontonoz et al., 1994, 1995). Although we have shown that EETs, DHETs, and 20-HETE activate PPARγ, the expression of CYP4A and CYP2C enzymes responsible for their formation have not been described in adipocytes. Therefore, the biological effects of P450 eicosanoids in adipocytes are not clear.

PPARα is well established as a mediator of hepatic lipid homeostasis. Because many studies have demonstrated the responsiveness of hepatic P450s to fibrate treatment, the biological consequences of increased P450s in the liver have not been elucidated. Through activation of PPARs, P450 metabolites of arachidonic acid after apoA-I and apoA-II expression in hepatocytes and may play a role in regulating triglyceride transport. It will be interesting to examine the overall effect of P450 eicosanoids on fatty acid transport and metabolism by analyzing a panel of genes involved in these processes. P450 levels are often altered in model systems of hypertension and diabetes. Because P450 eicosanoids may also be modulated autoregulatively by inducing PPARα-responsive P450s and sEH, it may be of therapeutic benefit to modify their levels by targeting PPARα.

Acknowledgments. We acknowledge the help of Colleen Hefferin at the University of California, San Francisco (UCSF) Liver Center for the preparation of primary hepatocytes and Dr. David Ginzing of the UCSF Cancer Center for help with the design of primers and probes used in quantitative reverse transcriptase-PCR experiments.

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