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Cytochrome P450 Eicosanoids are Activators of Peroxisome Proliferator-Activated Receptor Alpha (PPARα)

Valerie Y. Ng, Yong Huang, L. Manmohan Reddy, John R. Falck, Emil T. Lin, and Deanna L. Kroetz

Department of Biopharmaceutical Sciences (V.Y.N., Y. H., E.T. L., D.L.K) and the Liver Center (D.L.K.), University of California, San Francisco, and the Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas (M.R., J.R.F.)

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Running Title: CYP eicosanoids activate PPARα

Corresponding author: Deanna L. Kroetz, Ph.D., Department of Biopharmaceutical Sciences, University of California San Francisco, 1550 4th Street, Box 2911, San Francisco, CA 94143-2911; Tel: (415) 476-1159; Fax: (415) 514-4361; Email: deanna.kroetz@ucsf.edu

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CYP, cytochrome P450; HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; sEH, soluble epoxide hydrolase; DHET, dihydroxyeicosatrienoic acid; K_{Ca}, calcium-activated potassium; PPAR, peroxisome proliferator-activated receptor; apo, apolipoprotein; PPRE, peroxisome proliferator response element; T_3, 3,3´,5-triiodo-L-thyronine; CPT, carnitine palmitoyl transferase

ABSTRACT

Cytochrome P450 (CYP) 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 epoxyeicosatrienoic acid (EET) degradation to the dihydroxyeicosatrienoic acids (DHETs), are induced upon activation of peroxisome proliferator-activated receptor alpha (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 CYP 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, CYP eicosanoids induced CYP4A1, sEH, and CYP2C11 expression, suggesting that they can regulate their own levels. Given that CYP eicosanoids have multiple cardiovascular effects, pharmacological modulation of their formation and/or degradation may yield therapeutic benefits.

INTRODUCTION

Eicosanoids generated from arachidonic acid metabolism by cytochrome P450 (CYP) enzymes are important autocrine and paracrine factors that have diverse biological functions. CYP eicosanoids are involved in the regulation of vascular tone, renal tubular transport, cardiac contractility, cellular proliferation, and inflammation (Roman, 2002). The major products of CYP-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; Wu et al., 1997). EETs are subsequently metabolized by the soluble epoxide hydrolase (sEH) into dihydroxyeicosatrienoic acids (DHETs) (Zeldin et al., 1993; Yu et al., 2000).

Regulation of CYP eicosanoid levels is determined by many factors, which includes the induction or repression of the CYP enzymes responsible for their formation. Large numbers of studies have focused on the induction of rat CYP4A protein and mRNA levels by anti-hyperlipidemic agents such as clofibrate (Kimura et al., 1989). Recently, 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 are 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 the peroxisome proliferator-activated receptor alpha

(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 upregulation of apolipoprotein (apo) A-I and apo A-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 (Fruchart et al., 2001). In human hepatocytes however, apo A-I expression is decreased following PPARα activation (Vu-Dac 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-Dac 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 are all inhibited after PPARα activation (Bishop-Bailey, 2000).

Mechanistically, PPARs are ligand-activated transcription factors which, 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 B_4 also activate PPARα (Devchand et al., 1996). A 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 CYPs and sEH are regulated by peroxisome proliferators, we hypothesize that CYP eicosanoids are also agonists of PPARα. To test whether CYP 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, CYP eicosanoids were shown to induce the binding of PPARα to a PPRE. Furthermore, we show that 11,12-EET and 14,15-DHET behave like peroxisome proliferators in that they were able to alter apoA-I and apoA-II mRNA expression in primary rat hepatocytes. Since apoA-I and apoA-II are involved in the transport of HDL, these findings suggest that CYP eicosanoids may play a role in the regulation of triglyceride levels via PPARα. In addition, we show that CYP and sEH mRNA levels were increased in primary rat hepatocytes after treatment with these eicosanoids. These results suggest that CYP eicosanoids may regulate their own levels through a complex autoregulatory mechanism.

MATERIALS AND METHODS

Materials. Wy 14,643 and ciglitazone were obtained from Biomol (Plymouth Meeting, PA) and eicosanoids were purchased from Cayman Chemical (Ann Arbor, MI). The EET agonist 11,12-epoxyeicosa-8(Z)-enoic acid (11,12-EEZE) was synthesized as described previously (Falck et al., 2003). 3,3´,5-Triiodo-L-thyronine (T_3) was purchased from Sigma Chemical Company (St. Louis, MO). CYP4A1 and CYP2C11 primary antibodies were purchased from Gentest (Woburn, MA). The sEH antibody was a kind gift from Dr. Bruce Hammock (University of California, Davis), and the CYP2C23 antibody was a kind gift from Dr. Jorge Capdevila (Vanderbilt University). Gal4 expression and reporter plasmids were provided by Dr. Thomas Scanlan (University of California, San Francisco) and pCMV-mPPARα and pRS-hRXRα plasmids were a generous gift from Dr. Ronald Evans (Salk Institute, La Jolla, CA).

Cell Culture. HepG2 cells were obtained from American Type Cell Culture 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 non-essential amino acids, and penicillin-streptomycin. Sprague Dawley primary hepatocytes were isolated by the UCSF Liver Center Core Facility, were cultured on Collagen Type I plates (BD Biosciences) and overlaid with 0.25 mg/ml Matrigel (BD Biosciences, Bedford, MA) in HCM™ medium (Cambrex, Walkersville, MD). Primary hepatocytes were treated on the third day after isolation. All cells were cultured in 37°C with 5% CO_2.

Transactivation Assays. HepG2 cells were plated in 24-well plates at $8x10^4$ cells per well and transfected the next day using Lipofectamine PLUS reagents (Invitrogen, Carlsbad, CA) with 0.1-1 ng of Gal4-hPPARα or Gal4-hPPARγ, 100 ng UAS_4-LUC, and 35 ng of pCMV-βgal.

After 24 hours, cells were dosed with 50 μM Wy 14,643, 10 μM ciglitazone, or 10 μM CYP eicosanoids in serum-free medium containing up to 0.1% DMSO for 6 hours. 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, Madison, WI). To determine β-galactosidase activity, cell lysates were incubated with the substrate *o*-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 CYP eicosanoids for 6 h. Extraction of lipids from culture medium and cells have been described previously (Fang et al., 2001). Briefly, 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 PBS, 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% H_2O 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 previously described (Yu et al., 2004). Briefly, analysis of lipids by high-performance liquid chromatography (HPLC) was performed using a 4.6 x 150 mm 5μm Luna C18(2) column (Phenomenex, Torrance, CA) and

lipids were eluted from the reverse-phase HPLC column. The effluent was then injected into a Quattro Ultima tandem-quadrupole mass spectrometer (Micromass, Manchester, UK) and subjected to negative mode electrospray ionization (ESI). Multi-reaction monitoring (MRM) was employed to detect the eicosanoids simultaneously, and data were analyzed using the MassLynx 3.5 software (Milford, MA).

Gel Shift Assays. pCMX-mPPARα (1 μg) and pRS-hRXRα (1 μg) were translated using the TNT reticulocyte in vitro translation system (Promega, Madison, WI). The binding reaction contained 1 μl of translated PPARα, 0.3 μl of RXRα in 10 mM Tris, 150 mM KCl, 6% glycerol, 0.05% Igepal, 1 mM DTT, 2 μg poly (dI-dC), and CYP eicosanoids with or without 1 μl PPARα or RXRα antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The reactions were incubated for 10 min on ice before 250,000 cpm [γ-^{32}P]-labeled PPRE was added. The sequence for the consensus PPRE oligonucleotide is 5′-CAA AAC TAG GTC AAA GGT CA-3′, and the sequence for the mutant oligonucleotide is 5′-CAA AAG TAG CAC AAA GCA CA-3′. The oligonucleotides were end-labeled with [γ-^{32}P] using T4 polynucleotide kinase. Following 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 (Amersham Biosciences, Piscataway, NJ).

Real-Time Quantitative PCR. Primary hepatocytes were treated with peroxisome proliferators or eicosanoids for 24 to 48 hours. RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription is carried out using M-MLV (Promega, Madison, WI) reverse transcriptase. Primers and probe sets were designed using Primer Express (Applied Biosystems, Foster City, CA) and are listed in Table 1. Probes were labeled with reporter dye, 6-carboxy-fluorescein phosphamidite (FAM) at

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the 5′-end and the dye quencher, Black Hole Quencher at the 3′-end. The apoA-I, apoA-II, and CPT1A primer and probe sets were Assays-on-Demand purchased from Applied Biosystems (Foster City, CA). Reactions were run on an ABI Prism 7700 and cycling conditions were: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The relative expression of specific transcripts was calculated by the following formula: Relative expression = $2^{-\Delta\Delta Ct}$ where $Ct = (\Delta Ct_{target} - \Delta Ct_{cyclophilin})_{treated} - (\Delta Ct_{target} - \Delta Ct_{cyclophilin})_{control}$.

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.

RESULTS

CYP eicosanoids transactivate PPARs. Transactivation assays were carried out to determine whether EETs or DHETs were able to functionally activate PPARα and PPARγ (Figure 1). HepG2 cells were transfected with a Gal4 plasmid containing the ligand-binding domain of either human PPARα or PPARγ (Gal4-hPPARα or Gal4-hPPARγ), and a luciferase reporter plasmid containing 4 repeats of the Gal4 response element (UAS_4-LUC). In cells not transfected with hPPARα or hPPARγ, only minimal basal activation was observed. When hPPARα-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 (Figure 1A). The highest activation was observed with 11,12-EEZE, a monounsaturated 11,12-EET analog. 8,9-EET did not significantly activate either hPPARα or hPPARγ.

The effects of DHETs on hPPAR activation were also examined (Figure 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 (Figure 1C). To exclude the possibility that the activation observed with CYP eicosanoids was non-specific for nuclear receptors, we tested their ability to transactivate an unrelated receptor, the thyroid hormone receptor beta (TRβ) (Figure 1D). The endogenous

ligand of TRβ, triiodo-L-thyronine (T_3) 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 hPPARα and hPPARγ in a dose-dependent and saturable manner (Figure 2). Cells were transfected as described above and treated with 0.1 μM to 100 μM 14,15-DHET. A steep dose-dependence was observed between 1 μM and 10 μM of 14,15-DHET. Maximal activation was observed at 50 μM for both receptors with EC_{50} values of 1.5 μM and 2.5 μM for hPPARα and hPPARγ, respectively.

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 CYP 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.

EETs and DHETs induce PPAR/RXR binding to a PPRE. Gel shift assays were carried out to determine whether CYP 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 (Figure 3A). Supershifts with mPPARα and RXRα specific antibodies demonstrated that heterodimers consisted of mPPARα and RXRα (Figures

3B-3E). 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 (Figure 3F). Likewise, 20-HETE induced mPPARα/RXRα-specific heterodimerization and subsequent binding to the PPRE (Figure 3G).

CYP eicosanoids alter the expression of PPARα-responsive genes. After demonstration of PPARα activation by CYP eicosanoids, it was of interest to investigate whether CYP 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 carnitine palmitoyl transferase 1A (CPT1A) was determined by real time quantitative PCR (Figure 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 apoAII 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 CYP eicosanoids, in contrast to potent activation by Wy 14,643.

Since peroxisome proliferators can modulate CYP and sEH levels, we investigated whether CYP eicosanoids had the ability to modify CYP and sEH levels, thereby regulating their own expression (Figure 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-DHET and 20-HETE. Interestingly, CYP eicosanoids were more potent than Wy 14,643 in inducing CYP2C11 expression, with 20-HETE being the most

effective inducer, resulting in a 8-fold increase of CYP2C11 mRNA. CYP2C23 mRNA was increased only 1.5-fold after treatment with Wy 14,643 and 11,12-EET.

DISCUSSION

CYP 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 CYP 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, endogenous EET levels in rodent plasma (Yu and Kroetz, unpublished results) and human liver (Karara et al., 1991) are in the nanomolar range. The ED_{50}s 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 following entry into the cell. The above phenomenon makes it difficult to determine 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 CYP eicosanoids may be preferentially metabolized in the cell, thus influencing PPAR activation. Differences in PPARα activation by regioisomeric EET/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 urea-based and chalcone oxide sEH inhibitors was not possible in these studies since these inhibitors also activated PPARs (Ng and Kroetz, unpublished data and 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 PPARδ (Forman et al., 1997), and palmitic, oleic, linolenic, and arachidonic acids are agonists for murine PPARα and PPARγ (Kliewer et al., 1997). Recently, 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) show that these have beneficial effects on insulin resistance and display antihyperglycemic and hypolipidemic activities. Since CYP eicosanoids are endogenous compounds that are released into the intracellular environment, it is of interest to explore whether CYP eicosanoids play a role in improving insulin sensitivity and maintaining lipid homeostasis.

It was recently shown that 10-20 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-hPPARα more than 10-fold, suggesting that the non-ω-hydroxylated EETs may be more potent activators of PPARα. It is important however, to take into account the differences in cell type and the species of PPARα that were employed 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_is for EETs and Wy 14,643 are about 10- and 26-fold higher, respectively (Cowart et al., 2002). Based on ligand displacement assays, EETs and Wy 14,643 are expected to have lower affinity for PPARα than 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-mPPARα 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 CYP 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(S)-HETE, 15-deoxy-$\Delta^{12,14}$-PGJ_2, and hydroxylated EETs are naturally occurring eicosanoid ligands for PPARα and PPARγ (Forman et al., 1997; Kliewer et al., 1997; Cowart et al., 2002). Given the similarity in structure between these established ligands and CYP eicosanoids, and the promiscuous ligand binding pocket of PPARs, it is not surprising that CYP 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 CYP 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 CYP eicosanoids can assume. Structural information will provide additional insight as to why certain CYP 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. CYP eicosanoids decreased apoA-I mRNA levels in primary rat hepatocytes to a similar or greater degree as the well-characterized PPARα agonist Wy 14,643. The regulation of the apoA-I gene by PPARα is species and ligand-specific (Vu-Dac et al., 1998). In previous rat hepatocytes studies, fenofibrate transcriptionally decreased apoA-I mRNA while benzafibrate, 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. Since CYP 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 the 20-HETE effect is not due solely to PPARα activation. The RXR homodimer (Nagasaki et al., 1994), Rev-erbα (Vu-Dac et al., 1998), HNF-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 CYP 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 CYP 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 of was induced 1.6-fold, whereas ω-1 activity decreased 38% in response to LPS 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 CYPs to LPS stimulation between Fischer and Sprague Dawley rats, the authors 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 CYP eicosanoids mediate their effects via other mechanisms.

The ability of CYP 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 Figure 5). CYP4A and sEH mRNA expression are 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 CYP eicosanoid treatment. One possibility for the minimal effect of CYP 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 PPARγ can also be upregulated (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 (Figure 5), whereas when hepatocytes were treated with CYP eicosanoids, the potentially opposing dual effects elicited by both PPARα and PPARγ may account 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 and CYP 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 CYP 4A and sEH, CYP2C11 expression was markedly induced following treatment with CYP eicosanoids. Of note is that the CYP eicosanoids are more potent in inducing CYP2C11 than Wy 14,643. It has previously been reported that dietary administration of Wy-14,643, gemfibrizol 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 following 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 where 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 downregulation 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 while 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 apo-AI and apo-AII, the route of agonist exposure may also play an important role in the effects 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 aP2 and PEPCK (Tontonoz et al., 1994; Tontonoz et al., 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 CYP eicosanoids in adipocytes are not clear.

PPARα is well established as a mediator of hepatic lipid homeostasis. Although many studies have demonstrated the responsiveness of hepatic CYPs to fibrate treatment, the biological

consequences of increased CYPs in the liver have not been elucidated. Through activation of PPARα, CYP metabolites of arachidonic acid alter 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 CYP eicosanoids on fatty acid transport and metabolism by analyzing a panel of genes involved in these processes. CYP levels are often altered in experimental models of hypertension and diabetes. Since CYP eicosanoids may also be moderately auto-regulatory by inducing PPARα-responsive CYPs and sEH, it may be of therapeutic benefit to modify their levels by targeting PPARα.

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FOOTNOTES

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Address reprint requests to:

Deanna L. Kroetz, Ph.D.

Department of Biopharmaceutical Sciences

University of California San Francisco

1550 4th Street, Box 2911

San Francisco, CA 94143-2911

Tel: (415) 476-1159

Fax: (415) 514-4361

Email: deanna.kroetz@ucsf.edu

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FIGURE LEGENDS

Figure 1. CYP 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 UAS_4-LUC. Wy 14,643 (Wy, 50 μM), ciglitazone (Cig, 10 μM), or *A*, EETs (10 μM), *B*, DHETs (10 μM), or *C*, 20-HETE (10 μM) 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 UAS_4-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 UAS_4-LUC). The values shown are the mean ± SD of a representative experiment performed in quadruplicate. Similar results were obtained in additional experiments. * Significant difference compared to DMSO ($p<0.05$).

Figure 2. Dose dependent transactivation of hPPARα and hPPARγ by 14,15-DHET.

Transactivation assays were performed as described in Figure 1. Transactivation of *A*, hPPARα and *B*, hPPARγ by 0.1-100 μM 14,15-DHET is shown in the absence and presence of hPPARα or hPPARγ. The values shown are mean ± SD of a representative experiment performed in quadruplicate. EC_{50} values of 1.5 μM for hPPARα and 2.5 μM for hPPARγ were estimated using GraphPad Prism.

Figure 3. CYP eicosanoids induce mPPARα/RXRα-PPRE binding. EMSAs were performed by incubating *in vitro* translated mPPARα and RXRα with DMSO control, 5 μM Wy 14,643, or 1 μM CYP eicosanoids and ^{32}P-labeled PPRE. The complex was resolved through a

5% non-denaturing 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 non-specific binding. Competition assays were performed with increasing amounts of cold PPRE (*F* and *G*).

Figure 4. CYP eicosanoids induce PPARα-responsive genes in rat hepatocytes. Primary hepatocytes from Sprague Dawley rats were treated for 24 h with DMSO, 50 μM Wy, 10 μM 11,12-EET, 14,15-DHET, or 20-HETE. Total RNA was isolated with Trizol reagent and RNA expression was determined by real-time quantitative PCR. Expression of apoA-I, apoA-II, CPT1A (A) and CYP4A1, sEH, CYP2C11, and CYP2C23 (B) are normalized to cyclophilin levels and expressed as increase over DMSO control. The values shown are mean ± SD of triplicate determinations. * Significant difference compared to DMSO ($p<0.05$).

Figure 5. Relationship between PPARα, CYPs, sEH, and eicosanoids. Schematic of the results from this and other studies showing the metabolism of arachidonic acid by CYPs and sEH. In this study, PPARα is activated by 20-HETE, EETs, and DHETs (depicted with bold arrows). PPARα may in turn regulate the expression of the enzymes responsible for the formation of these eicosanoids, resulting in a feedback mechanism (indicated by curved arrows). In the present study, CYP4A and sEH gene expression were moderately increased whereas CYP2C11 expression was markedly elevated following PPARα activation by CYP eicosanoids (enzymes examined are underlined).

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Table 1. Primers and probes used in quantitative RT-PCR

Rat cyclophilin	
Forward Primer	5′-CGA TGA CGA GCC CTT GG-3′
Reverse Primer	5′-TCT GCT GTC TTT GGA ACT TTG TC-3′
Probe	6FAM-CGC GTC TGC TTC GAG CTG TTT GCA-BHQ
Rat CYP4A1	
Forward Primer	5′-TCA CCT CCC TTC CAC TGG TT-3′
Reverse Primer	5′-TCC ACA CAT GTC ATA ATT TGC T-3′
Probe	6FAM-TCA CCT TGA AAC TGC TTG TGC CCA-BHQ
Rat sEH	
Forward Primer	5′-CTC TAA ACT GGT ATC GAA ACA CAG AAA G-3′
Reverse Primer	5′-ATG TCC TTC TCA GCT GTG ACC AT-3′
Probe	6FAM-CGT TGG GAA GGA AGA TCT TGG TCC CT-BHQ
Rat CYP2C11	
Forward Primer	5′-GCC TTG TGG AGG AAC TGA GG-3′
Reverse Primer	5′-AGC ACA GCC CAG GAT AAA GGT-3′
Probe	6FAM-AGC AAA GGT GCC CCT TTT GAT CCC-TAMRA
Rat CYP2C23	
Forward Primer	5′-TTC GGG CTC CTG CTC CTT A-3′
Reverse Primer	5′-CGT CCA ATC ACA CGG TCA AG-5′
Probe	6FAM-AGA GGT GCA AGC CAA AGT TCA TGA GGA-BHQ

Sequences were designed using Primer Express.

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Table 2. Analysis of CYP eicosanoid levels in HepG2 cells

	Extracellular	Free Intracellular	Lipid Bound Intracellular
11,12-EET Treatment[a]			
11,12-EET	32.1 ± 13.5%	ND	19.8 ± 2.97%
11,12-DHET	47.3 ± 7.57%	0.12 ± 0.013%	0.69 ± 0.15%
14,15-DHET Treatment[b]			
14,15-DHET	99.9 ± 34.0%	0.0015 ± 0.0001%	0.0145 ± 0.0045%

HepG2 cells were exogenously treated with 10 μM 11,12-EET or 14,15-DHET for 6 h. Cells and culture medium were collected and lipids were extracted and analyzed using liquid chromatography tandem mass spectrometry.

[a]The amount 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.

[b]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.

Figure 1

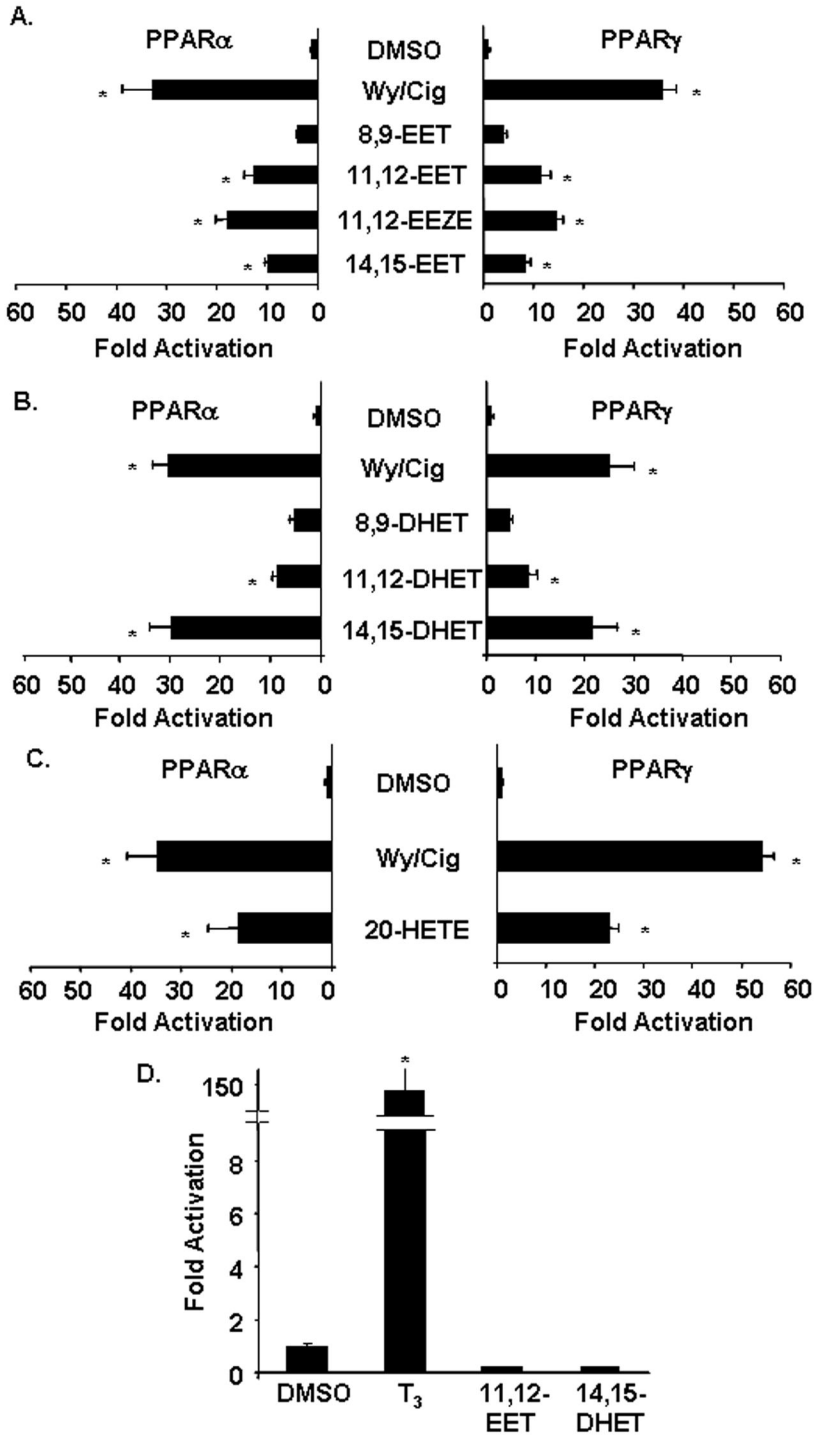
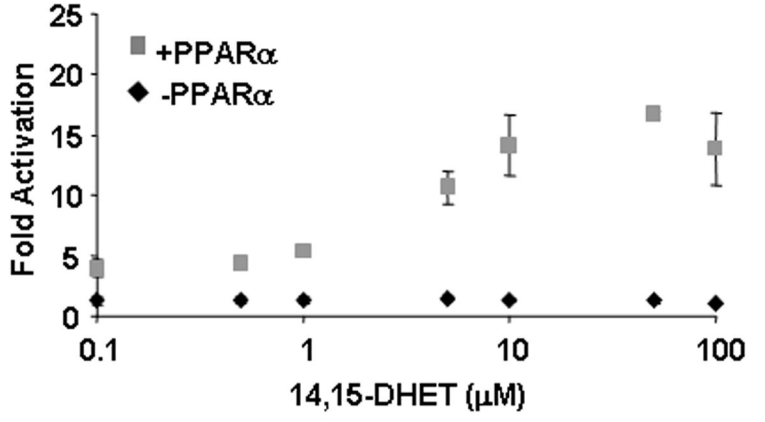

A.
PPARα
DMSO
PPARγ
Wy/Cig
8,9-EET
11,12-EET
11,12-EEZE
14,15-EET
60 50 40 30 20 10 0
0 10 20 30 40 50 60
Fold Activation
Fold Activation
B.
PPARα
DMSO
PPARγ
Wy/Cig
8,9-DHET
11,12-DHET
14,15-DHET
60 50 40 30 20 10 0
0 10 20 30 40 50 60
Fold Activation
Fold Activation
C.
PPARα
DMSO
PPARγ
Wy/Cig
20-HETE
60 50 40 30 20 10 0
0 10 20 30 40 50 60
Fold Activation
Fold Activation
D.
150
8
6
4
2
0
Fold Activation
DMSO
T3
11,12-EET
14,15-DHET

Figure 2

A.


25
20
15
10
5
0
+PPARα
-PPARα
Fold Activation
0.1
1
10
100
14,15-DHET (μM)

B.

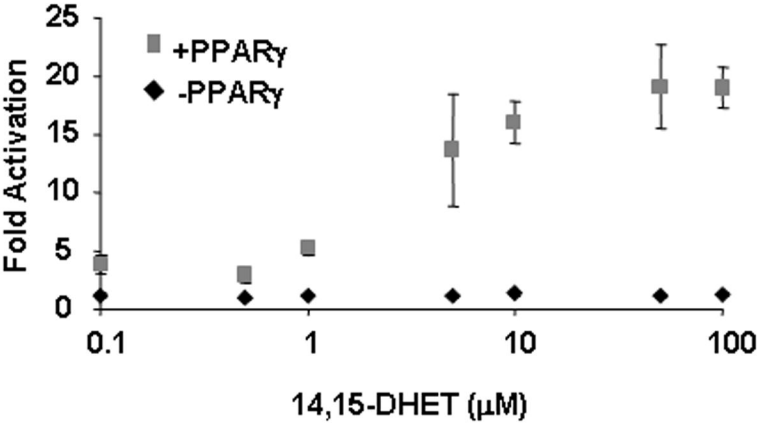

25
20
15
10
5
0
+PPARγ
-PPARγ
Fold Activation
0.1
1
10
100
14,15-DHET (μM)

Figure 3

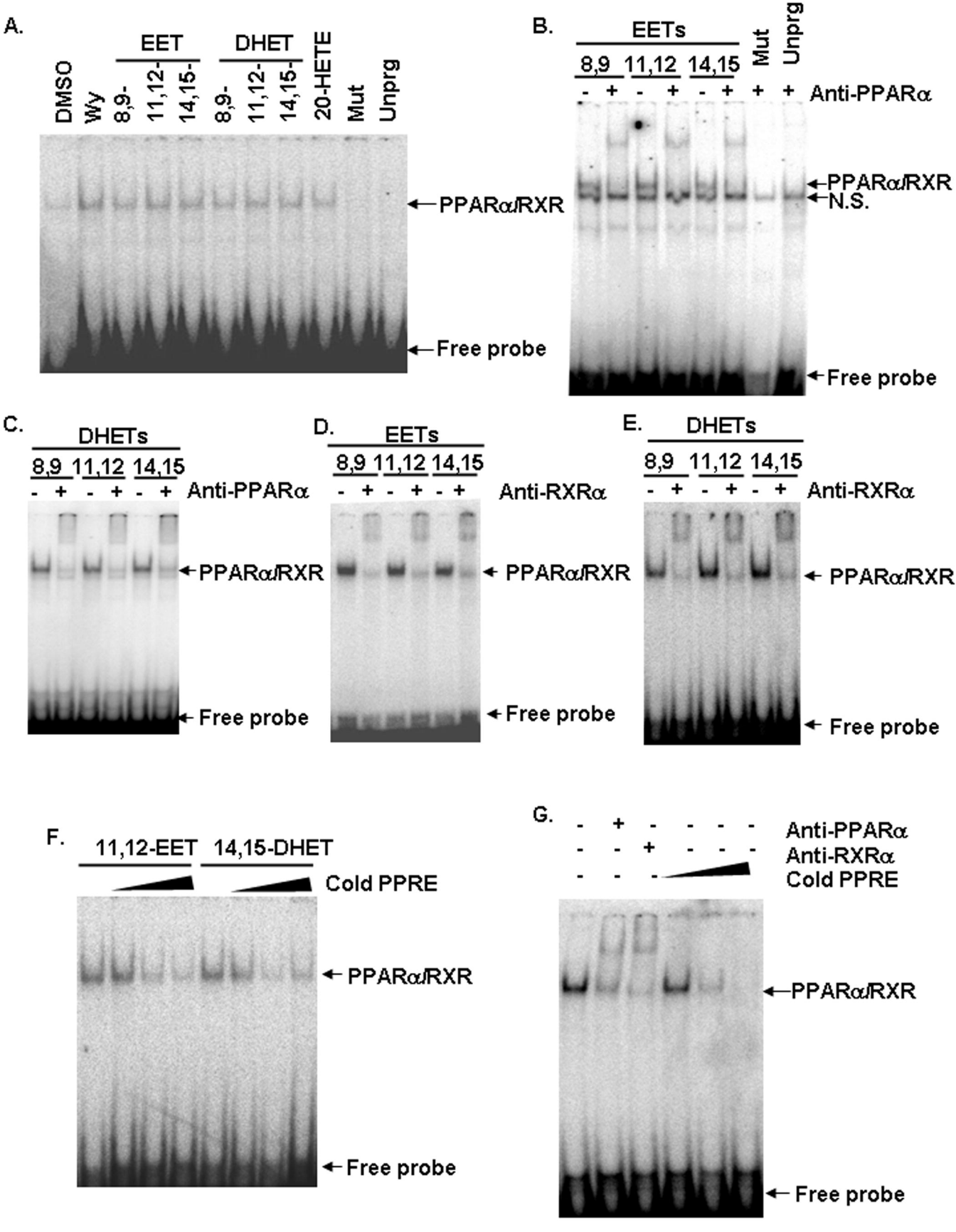

A.
DMSO
Wy
EET
8,9-
11,12-
14,15-
DHET
8,9-
11,12-
14,15-
20-HETE
Mut
Unprg
PPARα/RXR
Free probe
B.
EETs
8,9
11,12
14,15
Mut
Unprg
Anti-PPARα
PPARα/RXR
N.S.
Free probe
C.
DHETs
8,9
11,12
14,15
Anti-PPARα
PPARα/RXR
Free probe
D.
EETs
8,9
11,12
14,15
Anti-RXRα
PPARα/RXR
Free probe
E.
DHETs
8,9
11,12
14,15
Anti-RXRα
PPARα/RXR
Free probe
F.
11,12-EET
14,15-DHET
Cold PPRE
PPARα/RXR
Free probe
G.
Anti-PPARα
Anti-RXRα
Cold PPRE
PPARα/RXR
Free probe

Figure 4

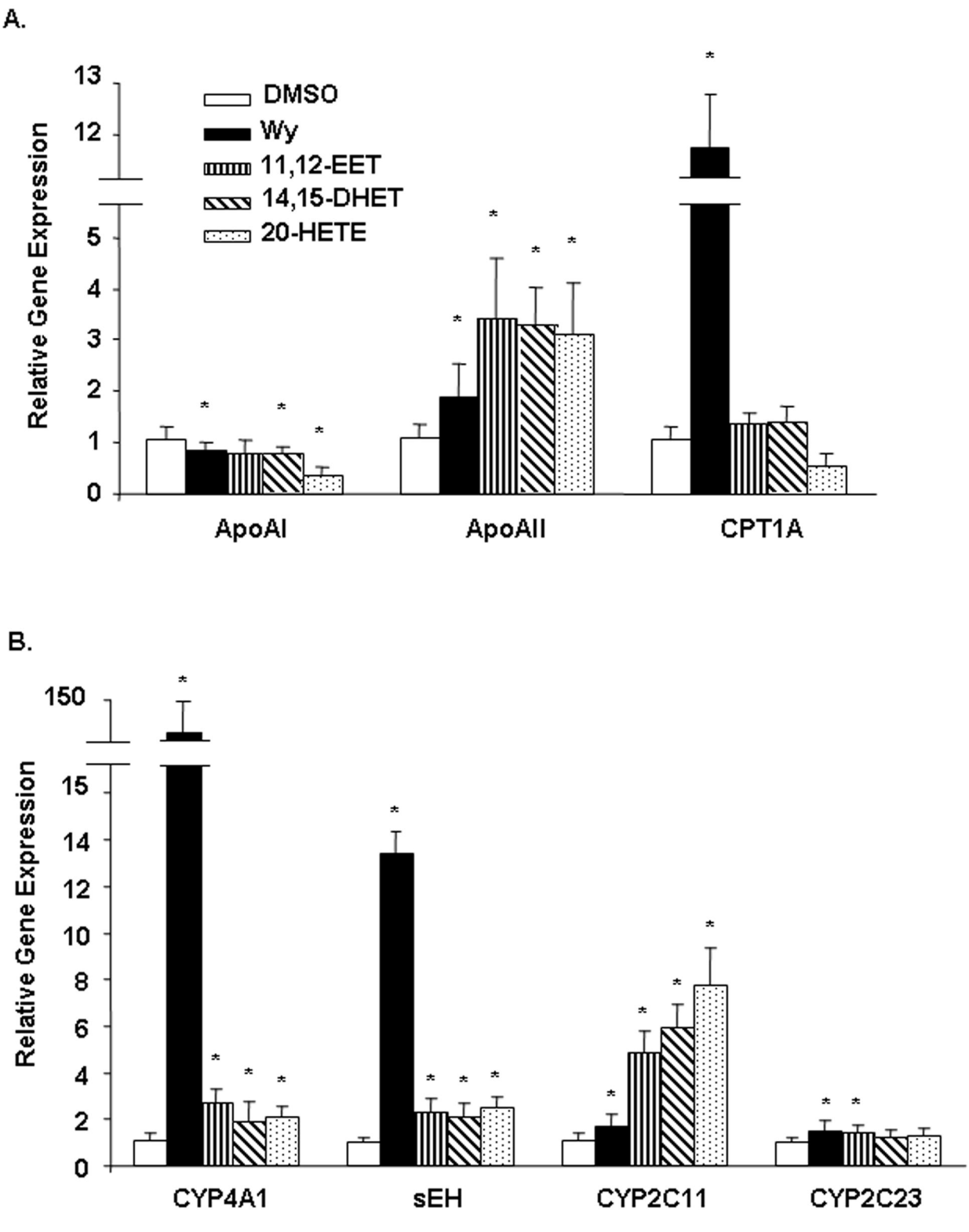
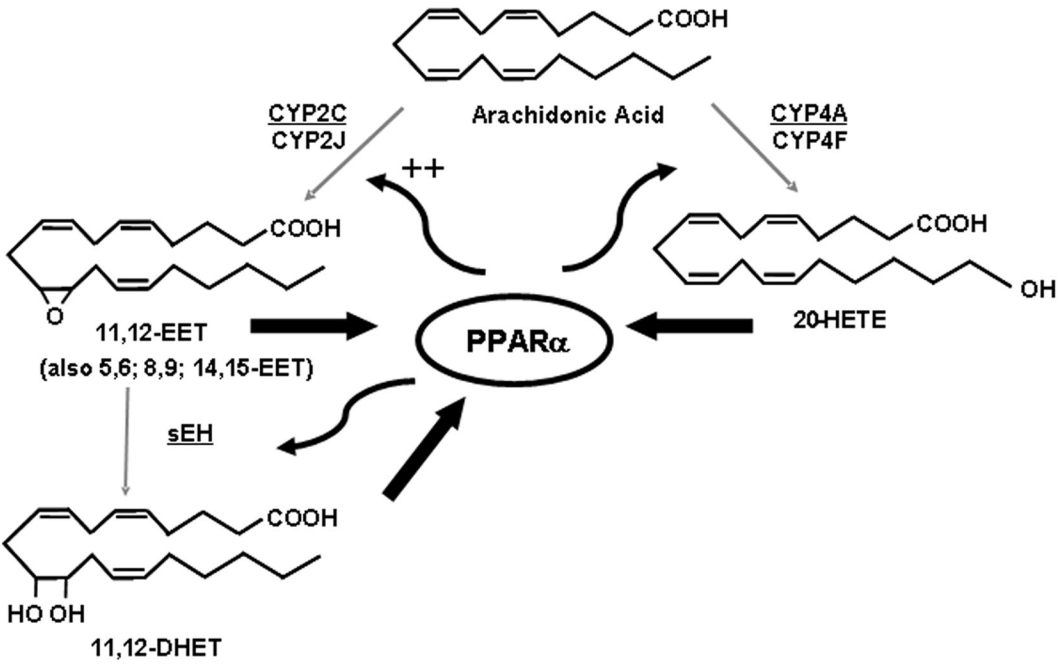

A.
Relative Gene Expression
13
12
5
4
3
2
1
0
DMSO
Wy
11,12-EET
14,15-DHET
20-HETE
ApoAI
ApoAII
CPT1A
B.
Relative Gene Expression
150
15
14
12
10
8
6
4
2
0
CYP4A1
sEH
CYP2C11
CYP2C23

Figure 5


COOH
Arachidonic Acid
CYP2C
CYP2J
CYP4A
CYP4F
++
COOH
O
11,12-EET
(also 5,6; 8,9; 14,15-EET)
COOH
OH
20-HETE
PPARα
sEH
COOH
HO OH
11,12-DHET