

P450 CYP2C epoxygenase and CYP4A ω -hydroxylase mediate ciprofibrate-induced PPAR α -dependent peroxisomal proliferation

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Abstract Peroxisomal proliferators, such as ciprofibrate, are used extensively as effective hypolipidemic drugs. The effects of these compounds on lipid metabolism require ligand binding activation of the peroxisome proliferator-activated receptor (PPAR) α subtype of nuclear receptors and involve transcriptional activation of the metabolic pathways involved in lipid oxidative metabolism, transport, and disposition. ω -Hydroxylated-eicosatrienoic acids (HEETs), products of the sequential metabolism of arachidonic acid (AA) by the cytochrome P450 CYP2C epoxygenase and CYP4A ω -hydroxylase gene subfamilies, have been identified as potent and high-affinity ligands of PPAR α in vitro and as PPAR α activators in transient transfection assays. Using isolated rat hepatocytes in culture, we demonstrate that specific inhibition of either the CYP2C epoxygenase or the CYP4A ω -hydroxylase abrogates ciprofibrate-induced peroxisomal proliferation, whereas inhibition of other eicosanoid-synthesizing pathways had no effect. Conversely, overexpression of the rat liver CYP2C11 epoxygenase leads to spontaneous peroxisomal proliferation, an effect that is reversed by a CYP inhibitor. **Based on these results, we propose that HEETs may serve as endogenous PPAR α ligands and that the P450 AA monooxygenases participate in ciprofibrate-induced peroxisomal proliferation and the activation of PPAR α downstream targets.**—Gatica, A., M. C. Aguilera, D. Contador, G. Loyola, C. O. Pinto, L. Amigo, J. E. Tichauer, S. Zanlungo, and M. Bronfman. **P450 CYP2C epoxygenase and CYP4A ω -hydroxylase mediate ciprofibrate-induced PPAR α -dependent peroxisomal proliferation.** *J. Lipid Res.* 2007. 48: 924–934.

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Several structurally unrelated xenobiotics with hypolipidemic properties induce peroxisome proliferation in the livers of rodents. These agents, termed hypolipidemic per-

oxisome proliferators (PPs), include an extensive number of synthetic compounds, such as ciprofibrate and related drugs used in the control of hyperlipemia, as well as environmental contaminants, such as plasticizers and herbicides (reviewed in Refs. 1, 2). In spite of their slight structural similarity, PPs as a group induce in rat and mouse a qualitatively similar pleiotropic response, consisting of hypolipidemia, hepatomegaly, proliferation of peroxisomes, and the induction of several hepatic enzymes involved in lipid metabolism (2–4). In addition, certain PPs are rapidly and abundantly transformed into non-metabolizable acyl-CoA thioesters in vitro and in vivo (5, 6), and significant changes are also produced in the levels of Coenzyme A (CoASH) and their fatty acyl-CoA thioesters (7, 8).

Long-term exposure to PPs leads to the development of hepatocellular carcinoma in rats and mice (9). PP effects are mediated by peroxisome proliferator-activated receptor (PPAR) α , as these effects are abrogated in PPAR α null mice (10). Fatty acids, and particularly their eicosanoid products, resulting from the lipoxygenase, cyclooxygenase, and P450 pathways, show high affinity for PPARs, and some of them have been suggested as endogenous PPAR α ligands (11–14). ω -Hydroxylated-eicosatrienoic acids (HEETs), products of the sequential metabolism of arachidonic acid (AA) by the cytochrome P450 CYP2C epoxygenase and CYP4A ω -hydroxylase gene subfamilies, have been identified as the ligands with the highest affinity

Abbreviations: AA, arachidonic acid; ADAPS, alkyl-dihydroxyacetone phosphate synthase; Ad-EPOX, adenoviral epoxygenase vector; Ad- β gal, adenoviral β -galactosidase vector; AOX, acyl-coenzyme A oxidase; CoASH, Coenzyme A; DDMS, *N*-methylsulfonyl-12,12-dibromododec-11-enamide; EET, epoxyeicosatrienoic acid; HEET, ω -hydroxylated-eicosatrienoic acid; HNF-4 α , hepatic nuclear factor-4 α ; PMP70, peroxisomal membrane protein; PP, peroxisome proliferator; PPAR α , peroxisome proliferator-activated receptor α ; PPOH, 6-(2-propargyloxyphenyl) hexanoic acid; PPRE, peroxisome proliferator response element.

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for PPAR α (14). These P450 isoforms play important physiological roles in the control of tissue and body homeostasis (15). *CYP2C* P450s catalyze the regioselective and enantioselective metabolism of AA to epoxyeicosatrienoic acids (EETs) and account for most of the epoxygenase activity in rat and human liver and kidney (16). EETs have vasoactive properties, modulate ion channels (17), and function as second messengers of pathways, such as epidermal growth factor-dependent signaling (18). EETs are excellent substrates for *CYP4A* isoforms (14), which selectively hydroxylate saturated and unsaturated fatty acids in the $\omega/\omega-1$ position (19–21). Some of the *CYP4A* isoforms are under the control of PPAR α , which regulates an adaptive *CYP4A* induction in response to starvation and diabetes (22). *Cyp4A* is also under the control of other physiological stimuli, including fatty acids and hormones (23).

We report here that inhibition of the *CYP2C* epoxygenase or the *CYP4A* ω -hydroxylase abrogates ciprofibrate-induced peroxisomal proliferation in isolated rat hepatocytes. Conversely, overexpression of the rat liver *CYP2C11* epoxygenase leads to spontaneous peroxisomal proliferation. Our data suggest that HEETs are endogenous PPAR α ligands and that the P450 AA monooxygenases participate in ciprofibrate-induced peroxisomal proliferation.

MATERIALS AND METHODS

Materials

Ciprofibrate was provided by the Sterling-Winthrop Institute. Williams E medium and FBS were obtained from GIBCO (Grand Island, NY). Indomethacin, lipoxygenase-specific inhibitors, and EET standards were from Cayman Chemical (Ann Arbor, MI). All other drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). PPAR α -specific antibodies were purchased from Affinity BioReagents (Golden, CO). Antibodies against the integral peroxisomal membrane protein (PMP70) and catalase were a kind gift from Dr. M. Santos (Department of Cellular and Molecular Biology, Faculty of Biological Sciences, P. Universidad Catolica de Chile). Antibodies against alkyl-dihydroxyacetone phosphate synthase (ADAPS) were a generous gift from Dr. W. Just (Biochemie-Zentrum, Ruprecht-Karls-Universität, Heidelberg, Germany).

Primary cultures

Rat hepatocytes were isolated as described (24). Cell viability was 80–90%, as determined by trypan blue exclusion. Isolated hepatocytes were seeded at 7.5×10^5 cells in 3.5 cm plastic dishes coated with collagen and cultured in Williams E medium supplemented with 5% FBS, as described previously (5). At 8 h, the medium was replaced by drugs containing medium. Inhibitor drugs were dissolved in DMSO, yielding a final DMSO concentration of 0.01%. For [^{14}C]AA metabolic experiments, cells were incubated for various times in medium containing 4 μM AA and 0.25 $\mu\text{Ci}/\text{dish}$ [^{14}C]AA (53 mCi/mmol; Perkin-Elmer Life and Analytical Science, Boston, MA). Cells were fixed with 1 ml of methanol containing 0.1% acetic acid and triphenylphosphine (0.1 mM) and extracted twice with 2 ml of chloroform, according to published methods (25). The combined organic phases were evaporated under argon and dissolved in 300 μl of 50% acetonitrile containing 0.1% acetic acid. Separation of [^{14}C]AA from its radioactive metabolites was performed by reverse-

phase HPLC with a 5 μm C18 symmetric 300 column (4 \times 250 mm; Waters Corp., Milford, MA) and a linear solvent gradient from initially acetonitrile-water-acetic acid (49.9:49.9:0.1, v/v/v) to acetonitrile-acetic acid (99.9:0.1, v/v) over 40 min at 1 ml/min (26). Fractions of 0.8 ml were collected, and their radioactivity was determined by liquid scintillation counting. Chemical synthesis of [^{14}C]14,15-EET was performed according to published methods and purified by reverse-phase HPLC (25, 27). Incubation of hepatocytes with [^{14}C]14,15-EET and separation from its metabolites were performed as described above for [^{14}C]AA. Cells were incubated with 4 μM 14,15-EET and 0.1 $\mu\text{Ci}/\text{dish}$ [^{14}C]14,15-EET.

RNA extraction and Northern blots

Nucleic acid hybridizations were performed using total rat hepatocyte RNA, which was isolated using the guanidinium-phenol method described previously (28) and quantified by standard spectrophotometric methods. Total hepatocyte RNA samples were fractionated by denaturing agarose electrophoresis and transferred to nylon membranes (Gene Screen Plus; NEN-PerkinElmer, Waltham, MA). Membranes were then cross-linked by UV irradiation and prehybridized in ULTRAHyb (Ambion, Austin, TX) for 2 h at 42°C. Hybridizations were done using specific cDNA probes for acyl-coenzyme A oxidase (AOX), peroxisomal membrane marker PMP70, and PPAR α . Probes were radiolabeled using Klenow amplification in the presence of [^{32}P] deoxythymidine. Hybridization was done at 48°C for 14 h. The probes were synthesized by PCR amplification of cDNAs. The following primers were used: for AOX, forward (5'-TGA-CAC-CAT-ACC-ACC-CAC-CAA-C-3') and reverse (5'-ACG-CAC-ATC-TTGGAT-GGC-AG-3'); for PMP70, forward (5'-TGT-GCG-GCT-CAC-TAG-ATA-CC-3') and reverse (5'-TAC-GAG-GAG-GAT-TGT-GGA-GG-3'); and for PPAR α , forward (5'-GTG-CCT-GTC-CGT-CGG-GAT-GT-3') and reverse (5'-GTG-AGC-TCG-GTG-ACG-GTC-TC-3').

Reporter expression assays, plasmids, and adenoviral infections

A reporter plasmid containing three tandem repeats of the peroxisome proliferator response element (PPRE) from the AOX gene, fused to the herpes virus thymidine kinase promoter upstream of the coding sequence for luciferase, and an expression vector containing murine PPAR α were a kind gift of Dr. R. M. Evans (Howard Hughes Medical Institute, Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, CA). The expression vector containing only the coding sequence for luciferase under the control of the herpes virus thymidine kinase promoter (Clontech, Palo Alto, CA) was used as a control. Transient transfections of CHO cells were carried out according to the manufacturer's instructions, using LipofectAmine 2000 (Life Technologies, Carlsbad, CA). CHO cells were grown in DMEM, supplemented with 4% FBS until 70–80% confluent, and transfected with 0.3 μg of reporter plasmid or cotransfected with the reporter plasmid, 0.2 μg of the PPAR α plasmid and 40 ng of a CMV- β -Gal vector (Clontech, Palo Alto, CA), for normalization. The recombinant adenovirus containing the predominant rat liver arachidonate epoxygenase (*CYP2C11*), fused to rat P450 oxidoreductase (Ad-EPOX) (29) and the bacterial β -galactosidase (Ad- β gal) transgenes, was under the control of the cytomegalovirus promoter. Antibodies against *CP2C11* and β -galactosidase, as well as *CYP2C* and *CYP4A* P450 inhibitors, were kindly donated by Dr. Jorge Capdevila (Department of Medicine, Vanderbilt University Medical School, Nashville, TN). Large-scale production of recombinant adenoviruses was performed from HEK 293 infected cells, as described (30). For adenoviral infections, primary

rat hepatocyte cultures were plated on 3.5 cm tissue culture dishes (200,000 total). Twenty-four hours after plating, cells were infected with 7×10^7 viral particles/cm². After 2 h of infection, unbound virus was removed and replaced with fresh medium. The cells were incubated at 37°C in 5% CO₂ for 48 h. Cells were then harvested and processed for blotting or immunocytochemistry.

RESULTS

Cytochrome P450 general inhibitors abrogate ciprofibrate-induced peroxisomal proliferation in cultured rat hepatocytes

As stated previously, eicosanoid products of the cyclooxygenase, lipoxygenase, and cytochrome P450 pathways have been proposed as endogenous PPAR α activators (11, 31–34). On this basis, we first determined whether inhibitors of these pathways had any effect on ciprofibrate-induced peroxisomal proliferation in isolated rat hepatocytes. We found that the CYP P450 general inhibitors, clotrimazole and ketoconazole (35), strongly decrease ciprofibrate-induced mRNA expression of the peroxisomal proliferation markers AOX and the peroxisomal membrane protein PMP70 (36) in a concentration-dependent manner, using GAPDH as a control (Fig. 1). Although GAPDH increases its expression upon long-term treatment of rats with some

PPs (37), we did not observe significant changes in its expression in our short-term (12 h) experiments. Under the same experimental conditions, neither the specific lipoxygenase inhibitors, caffeic acid [a 5- and a 12-lipoxygenase inhibitor (38)], baicalein [a 12-lipoxygenase inhibitor (39)], or sculetin [a 15-lipoxygenase inhibitor (40)], nor the selective cyclooxygenase inhibitor indomethacin (41), all in the 10–100 μ M concentration range, had any effect on the ciprofibrate-induced increase of peroxisomal proliferation markers (data not shown).

Selective inhibitors of cytochrome P450 isoforms CYP4A and CYP2C abrogate ciprofibrate-induced AOX and PMP70 mRNA expression without affecting PPAR α transcriptional activity or its mRNA expression and with minor or no effect on CYP isoform protein expression

Next, we used 6-(2-propargyloxyphenyl) hexanoic acid (PPOH) and *N*-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS), specific inhibitors of CYP4A-dependent ω -hydroxylation and CYP2C-dependent epoxidation, respectively (42), to dissect the P450 pathways involved in the inhibition of peroxisomal proliferation marker induction by ciprofibrate. Both inhibitors abrogate the ciprofibrate-induced expression of AOX and PMP70 in a concentration-dependent manner (Fig. 2A, B). To evaluate whether these inhibitors could act as direct antagonists of PPAR α ,

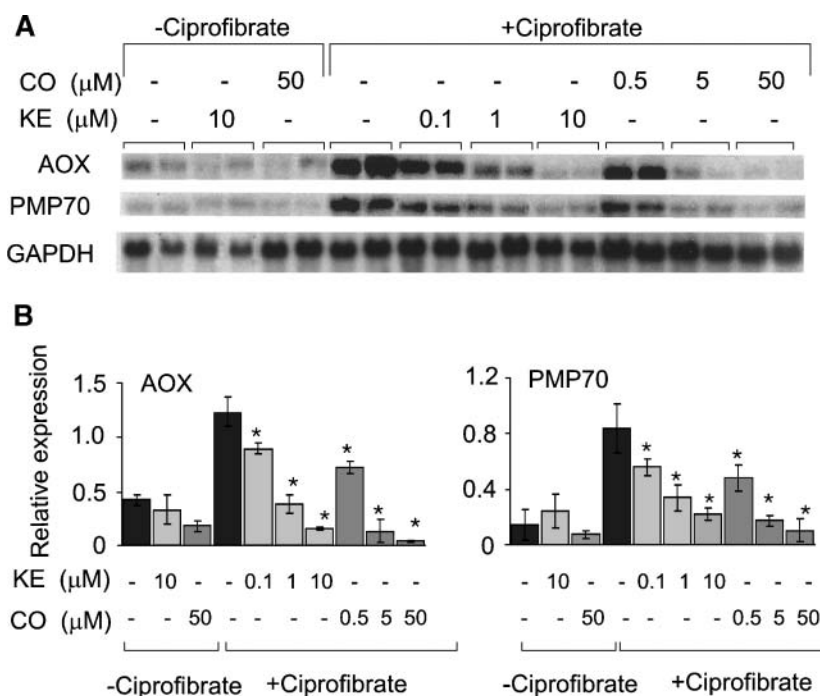


Fig. 1. Cytochrome P450 general inhibitors abrogate ciprofibrate-induced peroxisomal proliferation in cultured rat hepatocytes. Control and ciprofibrate-treated hepatocytes (100 μ M) were cultured for 12 h in the absence or presence of increasing concentrations of the general P450 inhibitors ketoconazole (KE) and clotrimazole (CO). The mRNA expression levels of acyl-coenzyme A oxidase (AOX) and peroxisomal membrane protein (PMP70) were evaluated by Northern blot. A: A representative Northern blot is presented, with duplicates from different culture dishes in each case. The expression of GAPDH was used as a control. B: Northern blots were quantified by scanning densitometry. Data represent means \pm SD of three independent experiments, corrected using GAPDH expression and expressed relative to mRNA expression in the absence of ciprofibrate and inhibitors. Asterisks indicate statistically significant differences from ciprofibrate-treated control cells ($P < 0.05$ or less).

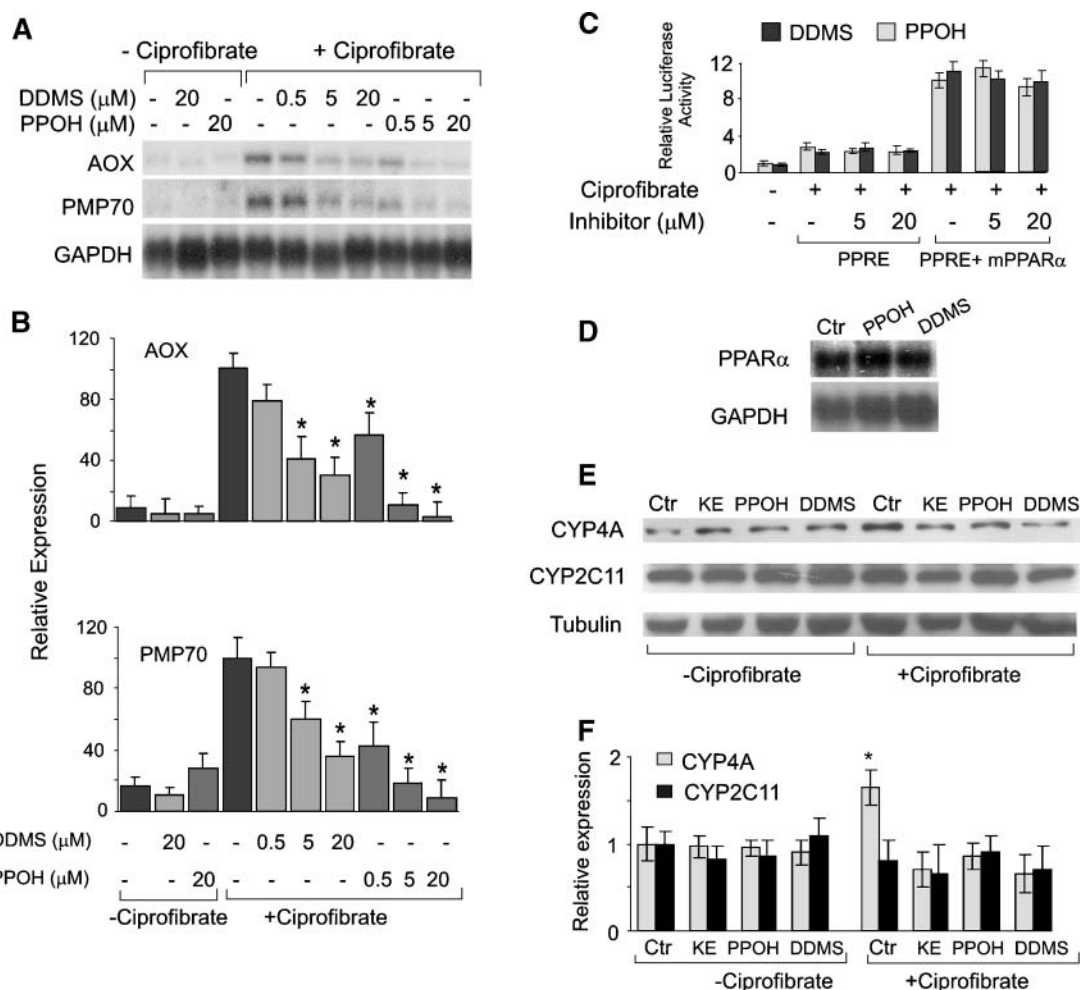


Fig. 2. Selective inhibitors of cytochrome P450 isoforms CYP4A and CYP2C abrogate ciprofibrate-induced AOX and PMP70 mRNA expression without affecting peroxisome proliferator-activated receptor α (PPAR α) transcriptional activity or its mRNA expression and with minor or no effect on CYP isoform protein expression. Hepatocytes were treated with or without 100 μM ciprofibrate for 12 h in the absence or presence of increasing concentrations of either 6-(2-propargyloxyphenyl) hexanoic acid (PPOH) or *N*-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS), specific inhibitors of ω -hydroxylation and epoxidation, respectively. **A:** A representative Northern blot is presented. **B:** Northern blots from three independent experiments were quantified by scanning densitometry for AOX (upper panel) and PMP70 (lower panel), corrected by the expression of GAPDH, and each one expressed relative to the corrected respective mRNA expression in ciprofibrate-untreated cells in the absence of inhibitors. Data represent means \pm SD. Asterisks indicate statistically significant differences from ciprofibrate-treated cells in the absence of inhibitors ($P < 0.05$ or less). **C:** CHO cells were transiently transfected with the peroxisome proliferator response element (PPRE)-luciferase reporter, or cotransfected with the PPRE reporter and mouse PPAR α (PPRE + mPPAR α), and treated for 24 h with 100 μM ciprofibrate in the absence or presence of 5 and 20 μM DDMS or PPOH. Luciferase activity was standardized relative to β -galactosidase (see Materials and Methods). Data represent means \pm SD of corrected luciferase activity from three independent experiments, expressed relative to the activity in the absence of ciprofibrate and inhibitors. **D:** Expression of PPAR α mRNA was assessed by Northern blot in hepatocytes treated for 12 h with 100 μM ciprofibrate in the absence (control) or presence of 20 μM DDMS or PPOH, as indicated. GAPDH mRNA expression was used as a control (Ctr). No effect of the drugs was found in PPAR α expression in two independent experiments. **E:** The expression of CYP2C and CYP4A was determined by Western blot in hepatocytes treated with 20 μM DDMS, 20 μM PPOH, or 10 μM ketoconazole (KE) in the absence and presence of ciprofibrate. A representative Western blot is presented. **F:** Western blots from three independent experiments were quantified by scanning densitometry for AOX CYP4A and CYP2C11, corrected by the expression of tubulin, and each one expressed relative to the corrected protein expression in ciprofibrate-untreated cells in the absence of inhibitors. Data represent means \pm SD. Asterisks indicate statistically significant differences from control cells. No significant changes were induced by the various treatments for CYP2C expression, whereas ciprofibrate induced CYP4A expression by 1.5- to 1.8-fold, an effect that was reverted to control values by CYP inhibitors.

we transiently cotransfected CHO cells with a PPRE-luciferase reporter plasmid and a mouse PPAR α expression plasmid (see Materials and Methods), and the transfected cells were treated for 24 h with 100 μM ciprofibrate in the presence or absence of increasing concentrations of DDMS

or PPOH. No effect on the relative ciprofibrate-induced luciferase activity was observed for either drug (Fig. 2C), showing that they do not affect PPAR α transcriptional activation. We used CHO cells because of the low efficiency of cultured hepatocytes in transfection experiments with the reporter

plasmid. Similarly, no effect of the drugs was found on PPAR α mRNA expression in isolated hepatocytes at the maximal concentration used (Fig. 2D).

Because both *CYP4A* and *CYP2C* are also regulated by PPAR α activators in opposite directions (43), we determined the levels of these proteins in control and ciprofibrate-treated cells in the absence and presence of *CYP* inhibitors (Fig. 2E; quantified in Fig. 2F). No effect of inhibitors was found in control cells for both proteins; however, in ciprofibrate-treated cells, *CYP4A* increased by 1.5- to 1.8-fold. Consistent with previous data, ciprofibrate-induced *CYP4A* upregulation was reversed by *CYP* inhibitors. Ciprofibrate and the inhibitors were without effect on *CYP2C* expression, either in the absence or presence of ciprofibrate. The lack of ciprofibrate effect on *CYP2C11* level is in agreement with published data showing that downregulation of *CYP2C* occurs only after 48 h of exposure of hepatocytes to the strong PP WY-14,643 (43). A short (12 h) exposure to ciprofibrate was used in our experiments, which also explains the low, although significant, effect of ciprofibrate on *CYP4A* expression. These results suggest that *CYP2C* and *CYP4A* activities are important for ciprofibrate-induced peroxisomal proliferation and that HEETs, the products of the sequential action of *CYP2C* and *CYP4A* on unsaturated fatty acids, may be the physiological activators of PPAR α in rat liver.

Putative hydroxy acid metabolites of AA, but not EETs, can be detected in control or ciprofibrate-treated hepatocytes metabolizing [1-¹⁴C]AA

To assess whether ciprofibrate induces measurable changes in AA oxidized metabolites, hepatocytes were incubated with [1-¹⁴C]AA to label only oxidized AA metabolites and not chain-shortened fatty acids, which are formed from AA as well as from EETs (44). Hepatocytes were incubated with [1-¹⁴C]AA for 1 and 6 h in the presence or absence of 100 μ M ciprofibrate. The labeled oxidized metabolites and the remaining [1-¹⁴C]AA were extracted with chloroform and resolved by reverse-phase HPLC, according to published methods (25, 26) (see Materials and Methods). AA is rapidly metabolized by hepatocytes, and only 30–35% of the radioactivity remained in the organic phase as [1-¹⁴C]AA after 1 h of incubation; however, after 6 h, only ~8% of radioactivity was still present as AA, as assessed by reverse-phase HPLC of the organic extracts (data not shown). No radioactivity was detected in the retention time corresponding to EETs, either in the presence or absence of ciprofibrate (Fig. 3), suggesting that EETs are very rapidly metabolized. For both incubation times, radioactivity was recovered mainly as [1-¹⁴C]AA and also in metabolites eluting earlier than EETs (peak X in Fig. 3A–C).

Radioactivity in peak X, which presents a retention time corresponding roughly to that of $\omega/\omega-1$ hydroxy acids, such as HEETs (26), increased 10–15% in ciprofibrate-treated cells. However, $\omega/\omega-1$ hydroxylation is one of the known routes for EET metabolism (45), and peak X probably contains other oxidized metabolites of AA. To indirectly assess whether this fraction may contain HEETs, hepatocytes were incubated for 1, 2, and 6 h in the pres-

ence of 14,15-[1-¹⁴C]EET in the absence or presence of 20 μ M of the *CYP4A* inhibitor PPOH. Labeled EET disappeared from the incubation medium even faster than AA in both conditions, and only 20% of the initial radioactivity eluted in the retention time of 14,15-EET after 1 h of incubation; after 6 h, it was <5%, as assessed by reverse-phase HPLC of the organic extracts (data not shown). After 1 h of incubation, no differences were induced by PPOH in the amount of radioactivity eluting with the EET peak. However, the radioactivity eluting in fractions around the retention time of peak X was reduced by almost 50% in the presence of PPOH (Fig. 3C). Quantification of the time course of radioactivity eluting in peak X in hepatocytes metabolizing 14,15-[1-¹⁴C]EET is presented in Fig. 3D. In all cases, PPOH induced a significant decrease in the radioactivity eluting in peak X, showing that fractions eluting in the peak X retention time are indeed sensitive to the *CYP4A1* inhibitor and that most of them probably contain HEETs.

The development of mass spectral methods for HEET quantification in biological samples and cultured cells, not yet available, will be necessary to directly assess and demonstrate that HEETs are present in peak X and whether ciprofibrate indeed increases HEET levels in hepatocytes after short-term incubation periods. Nevertheless, the results in this section partially answer two relevant points. First, they show that under our experimental conditions, EETs produced from AA are rapidly metabolized and their physiological concentration is probably maintained very low, making it unlikely that they act as PPAR α physiological ligands. This observation is in agreement with published data showing that in the liver free EETs represent <1% of total endogenous EETs (46). Second, although indirectly, the PPOH experiments suggest that HEETs are present in peak X and that PPOH indeed inhibits the formation of metabolic products from 14,15-[1-¹⁴C]EET, supporting the hypothesis that these eicosanoids are involved in PPAR α activation.

Adenovirus-mediated CYP2C11 overexpression induces peroxisomal proliferation in cultured rat hepatocytes

To further substantiate our previous conclusion, we infected cultured hepatocytes with a viral vector containing *CPY2C11*, the predominant liver epoxygenase, fused to rat P450 oxidoreductase (see Materials and Methods). Infected hepatocytes express the bifunctional protein (Ad-EPOX), as assessed by Western blot (Fig. 4A). Note that the antibody also recognizes endogenous *CP2C11* at the expected molecular mass, which is present in both uninfected and infected cells. Hepatocytes infected with the same viral vector but expressing β -galactosidase (Ad- β gal), to be used as controls, also showed increased expression of the β -galactosidase protein (data not shown). The levels of AOX and PMP70 mRNAs, as assessed by Northern blot, were increased by 4- and 6-fold, respectively, in hepatocytes overexpressing Ad-EPOX (Fig. 4B; quantified in Fig. 4C) compared with uninfected cells or cells infected with the Ad- β gal. A similar increase was observed in ciprofibrate-treated cells used as the positive control.

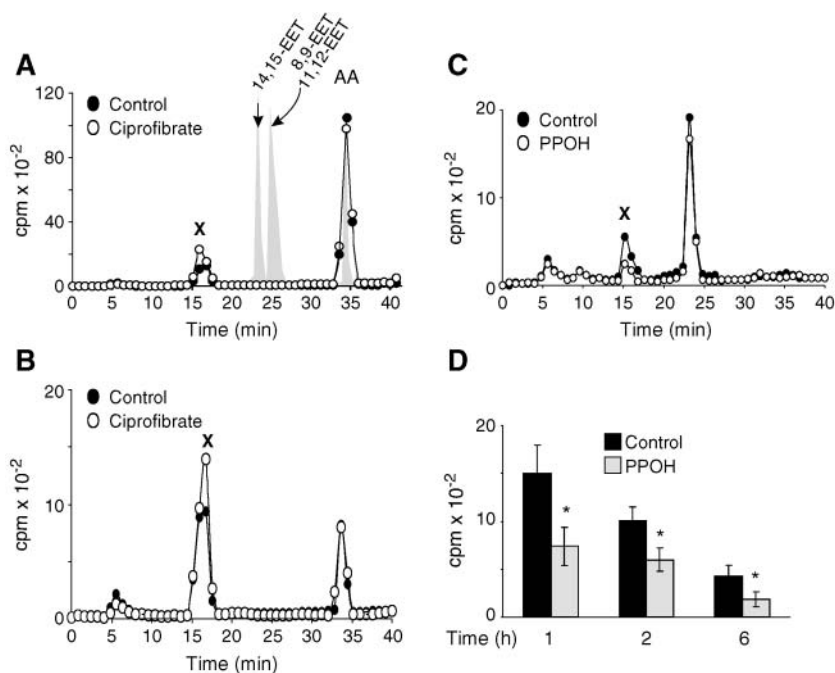


Fig. 3. Putative hydroxy acid metabolites of arachidonic acid (AA), but not epoxyeicosatrienoic acids (EETs), can be detected in control or ciprofibrate-treated hepatocytes metabolizing $[1-^{14}\text{C}]$ AA. Primary cultures of hepatocytes were incubated with $4\ \mu\text{M}$ AA and $0.25\ \mu\text{Ci}/\text{dish}$ $[1-^{14}\text{C}]$ AA for various times, fixed with methanol, and extracted with chloroform (see Materials and Methods). The organic phase was evaporated and resolved by reverse-phase HPLC. A: HPLC elution profile of organic extracts from cells incubated for 1 h with $[1-^{14}\text{C}]$ AA in the absence or presence of ciprofibrate. Under these conditions, $\sim 30\text{--}35\%$ of the initial radioactivity was present in the AA peak at the end of incubation, as assessed by reverse-phase HPLC. The retention times of EET standards and AA are presented in gray. No radioactivity eluting in the retention time of EET standards was detected after 1 h. A peak of radioactivity was detected at a retention time corresponding roughly to that of $\omega/\omega-1$ hydroxy acids (peak X; see text), which was increased slightly in ciprofibrate-treated cells. A representative chromatogram is presented. Similar results were obtained in three independent experiments. B: Cells were incubated for 6 h with $[1-^{14}\text{C}]$ AA, a condition at which only $\sim 8\%$ of the radioactivity remained as the $[1-^{14}\text{C}]$ AA peak at the end of the incubation. As in the 1 h experiment, no radioactivity eluted at the EET retention time, and ciprofibrate increased the radioactivity eluting in peak X. A representative chromatogram is presented. Similar results were obtained in two independent experiments performed with duplicates. C: Hepatocytes were incubated for 1 h in the presence of $4\ \mu\text{M}$ 14,15-EET and $0.1\ \mu\text{Ci}/\text{dish}$ $[1-^{14}\text{C}]$ 14,15-EET in the absence or presence of the *CYP4A1* inhibitor PPOH. After organic solvent extraction, labeled 14,15-EET was resolved from its metabolites by reverse-phase HPLC. Approximately 20% of the initial radioactivity was recovered in the 14,15-EET peak at the end of incubation. No changes were induced by PPOH in the radioactivity recovered with the 14,15-EET peak. However, PPOH induced a decrease in the radioactivity eluting with a retention time similar to peak X in experiments with $[1-^{14}\text{C}]$ AA. D: Time course of the radioactivity eluting in peak X in the absence or presence of PPOH. Data represent means \pm SD of the radioactivity eluting with peak X in triplicates of one experiment. In all cases, PPOH induced a significant decrease of radioactivity (* $P < 0.05$ or less). Similar results were obtained in a second independent experiment.

Next, we determined, by Western blot, the expression levels of peroxisomal proliferation marker proteins. We used ADAPS and catalase, together with PMP70 and *CYP4A* antibodies, as markers of peroxisomal proliferation. AOX was not used as a marker because of the unavailability of antibodies. All three peroxisomal proteins (36, 47, 48), as well as *CYP4A*, showed increased expression in Ad-EPOX-infected cells, ranging from 3-fold (catalase and ADAPS) to $\sim 5\text{--}6$ -fold (PMP70 and *CYP4A*), compared with control (no virus) or Ad- β gal-transfected cells (Fig. 5A; quantified in Fig. 5B). Ciprofibrate treatment also induced a significant increase in peroxisomal markers, as expected, but was lower than in Ad-EPOX-expressing cells for PMP70 and catalase. Furthermore, immunocytochemistry against

PMP70 and catalase showed the distinctive punctate pattern of peroxisomal markers (36), indicating a clear increase in peroxisome number in hepatocytes overexpressing Ad-EPOX compared with cells infected with Ad- β gal (Fig. 5C). Finally, we determined whether a *CYP* inhibitor could reverse the effect of *CYP2C11* overexpression. As shown in Fig. 5D (quantified in Fig. 5E), $1\ \mu\text{M}$ ketoconazole partially reversed the increased expression of PMP70 and *CYP4A* induced by *CYP2C11* overexpression, supporting a role of the *CYP* isoforms in PPAR α activation. We did not use higher ketoconazole concentrations, because some decrease in cell viability was observed after the 48 h culture period at concentrations of $>1\ \mu\text{M}$ ketoconazole.

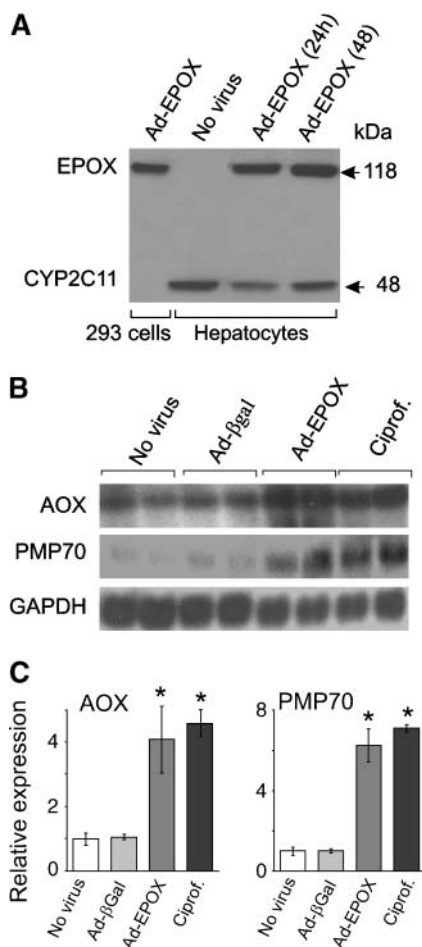


Fig. 4. Overexpression of the *CYP2C11* epoxygenase isoform induces increased expression of the epoxygenase protein and of AOX and PMP70 mRNAs in cultured rat hepatocytes. Primary cultures of rat hepatocytes were infected with an adenovirus containing the predominant liver epoxygenase *CYP2C11*, fused to rat P450 oxidoreductase (Ad-EPOX) or the β -galactosidase gene (Ad- β gal). **A:** Control of adenoviral transfections. Representative Western blot using antibodies against *CYP2C11* in HEK 293 cells (used for the large-scale production of recombinant adenoviruses; see Materials and Methods) infected with the *CYP2C11* recombinant adenovirus, uninfected hepatocytes (no virus), and infected hepatocytes after 24 and 48 h (Ad-EPOX). Molecular mass standards are shown at right. Expression of Ad-EPOX and of endogenous *CYP2C11* was observed at the expected molecular mass in Ad-EPOX-infected cells. Similar controls were performed for the expression of β -galactosidase in Ad- β gal-infected cells (not shown). **B:** Northern blot of AOX and PMP70 in control hepatocytes, hepatocytes infected with either the Ad- β gal or Ad-EPOX gene after 48 h of infection, or hepatocytes treated with 100 μ M ciprofibrate for 48 h (Ciprof.). GAPDH expression was used as a control. **C:** Upper panel, representative Northern blot. Lower panel, Northern blots from three independent experiments were quantified by scanning densitometry for AOX and PMP70, corrected by the expression GAPDH, and each one was expressed relative to control cells (no virus). Data represent means \pm SD. Asterisks indicate statistically significant differences from control cells ($P < 0.01$ or less).

DISCUSSION

This report provides evidence that *CYP2C* and *CYP4A* P450 isoforms play a role in the induction of peroxisomal proliferation and that their products are most likely endog-

enous PPAR α ligands in rat liver cells. The abrogation of ciprofibrate-induced peroxisomal proliferation by general and specific *CYP2C* and *CYP4A* inhibitors, but not of other eicosanoid-generating pathways, and their lack of effect on PPAR α expression or transcriptional activity suggest that HEET products of the sequential action of *CYP2C* and *CYP4A* on unsaturated fatty acids participate in, and apparently are necessary for, ciprofibrate-induced peroxisomal proliferation. Only minor or no changes in the expression of these isoforms were observed after short-term (12 h) exposure to ciprofibrate, a time period in which mRNA expression of PPAR α target genes is already increased. Moreover, the spontaneous peroxisomal proliferation observed in cells overexpressing *CYP2C11* suggests that these metabolites, by themselves, induce PPAR α activation.

This proposal is further supported by a previous report showing that HEETs are high-affinity PPAR α ligands, that they transactivate PPAR α in gene reporter assays, and that their precursors, the EET products of *CYP2C*, are excellent substrates for *CYP4A* (14). In addition, studies in the peroxisomal AOX null mouse, which exhibits massive peroxisomal proliferation, including *CYP4A* induction, show that increased intracellular concentration of fatty acid metabolites can induce peroxisomal proliferation (49). This view is further supported by studies in mouse nullizygous for both PPAR α and AOX that suggest a function of *CYP4A* isoforms in PPAR α signaling (50). The fact that we were unable to detect EET formation from [1- 14 C]AA under our experimental conditions suggests that EETs are rapidly metabolized and probably do not accumulate as free fatty acids in the liver. This result is consistent with previous data showing that endogenous liver EETs are present mainly as esters in phospholipids, which is a major pathway of EET metabolism (45, 46, 51), and that <1% is present as free fatty acids (46).

These considerations make it unlikely that EETs are involved directly in PPAR α activation. The ciprofibrate-induced slight increase in [1- 14 C]AA metabolites present in peak X and the partial decrease in the radioactivity eluting in that position induced by the *CYP4A1* inhibitor PPOH in cells metabolizing [1- 14 C]14,15-EET suggest that more polar metabolites, such as HEETs, might be responsible for PPAR α activation. However, because sensitive techniques to measure HEETs in cells are not yet available, we cannot exclude the possibility that other metabolites of AA are increased in ciprofibrate-treated hepatocytes. Other derivatives of EETs, products of soluble epoxide hydrolase and hydroxy-eicosatrienoic acids, are also activators of PPAR α (52). However, their in vitro affinity for PPAR α is in the micromolar range, and most of the hydroxy-eicosatrienoic acids that are formed by cells are released into the extracellular fluid (45). Additionally, the suppression of ciprofibrate-induced peroxisomal proliferation by *CYP2C11* and *CYP4A* inhibitors does not support a physiological role for hydroxy-eicosatrienoic acids in PPAR α activation. Nevertheless, soluble epoxide hydrolase, which is also a PPAR α target gene (53), may be physiologically relevant in the disposing of excess EETs under conditions in which EET and HEET degradation

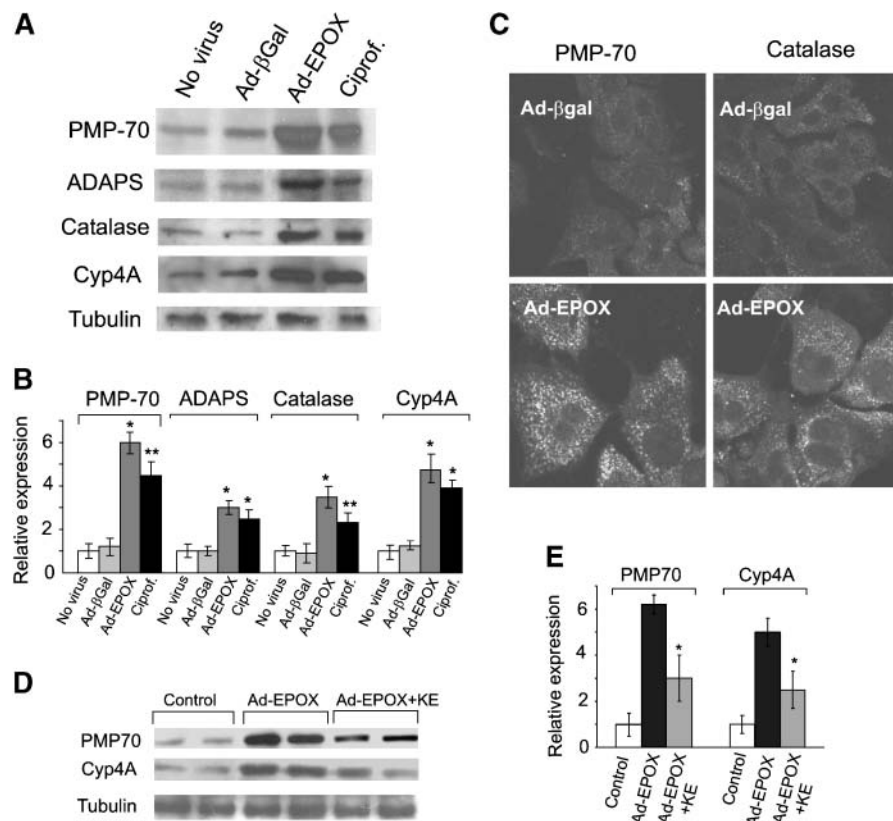


Fig. 5. Overexpression of the *CYP2C11* epoxygenase isoform induces the expression of peroxisomal proliferation marker proteins and increases peroxisome number in cultured hepatocytes. **A:** Western blots of PMP70, alkyl-dihydroxyacetone phosphate synthase (ADAPS), catalase, and *CYP4A* in control hepatocytes, hepatocytes transfected with the Ad-βgal or Ad-EPOX gene after 48 h of infection, or hepatocytes treated with 100 μM ciprofibrate for 48 h (Ciprof.). Tubulin was used as a control. **B:** Western blots from three independent experiments were quantified by scanning densitometry for PMP70, ADAPS, catalase, and *CYP4A*, corrected by the expression of tubulin, and each one expressed relative to control cells (no virus). Data represent means ± SD. Asterisks indicate statistically significant differences from control cells ($P < 0.01$); double asterisks indicate statistically significant differences from control cells and from cells overexpressing Ad-EPOX ($P < 0.05$ or less). **C:** Immunocytochemistry against PMP70 and catalase in cultured hepatocytes infected with either the Ad-βgal or the Ad-EPOX gene after 48 h of transfection, showing increased staining and peroxisome number in Ad-EPOX-transfected cells. **D:** *CYP2C11* epoxygenase-transfected hepatocytes were cultured for 48 h in the absence (Ad-EPOX) or presence of 1 μM ketoconazole (Ad-EPOX+KE). Ad-βgal-transfected cells were used as controls. Representative Western blots of PMP70 and *CYP4A*, which are highly induced in cells overexpressing the epoxygenase, are presented. Tubulin was used as a control. **E:** Quantification of three independent experiments as shown in D, corrected by the expression of tubulin, and each one expressed relative to control cells. Data represent means ± SD. Asterisks indicate statistically significant differences from Ad-EPOX cells ($P < 0.01$). A partial decrease in the peroxisomal target genes was observed at 1 μM ketoconazole. Higher ketoconazole concentrations were not used because they partially decreased cell viability after the 48 h culture period.

by peroxisomal β-oxidation is surpassed, as might occur under fasting conditions, in which fatty acid influx into the liver increases considerably.

Because *CYP4A* is a PPARα target gene, it is conceivable that its upregulation may contribute to the modulation of PPARα-dependent gene transcription and liver lipid homeostasis. Similarly, the downregulation of *CYP2C* after long-term exposure to PPs might also represent a feedback mechanism to decrease the synthesis of PPARα activators. There is extensive evidence for the function of *CYP2C* and *CYP4A* metabolites in cell signaling in extrahepatic tissues, including the modulation of ion channel activity and

transporters, mitogenesis, and the activation of tyrosine kinase cascades, mitogen-activated protein kinases, and phosphatidylinositol 3-kinase signaling pathways (51, 54), but whether these metabolites may activate similar pathways in liver cells is not known. As noted above, the identity and relative contribution of monooxygenase metabolites to PPARα-dependent peroxisome proliferation will have to await the development of sensitive techniques for HEET quantification in cells and tissues. Nevertheless, the present data add a new perspective from which to experimentally approach unresolved issues regarding the mechanism mediating PP effects as well as on PPARα functionality in

peroxisome proliferation-unresponsive species, such as humans (55, 56).

Although it is clear that PP-induced sustained PPAR α transcriptional activity is essential for PP-induced pleiotropic effects (11, 34, 57), contradictory observations persist when comparing the relative affinity of various PPs for PPAR α with its potency as a PP. The strong peroxisomal proliferator WY-14643 binds to PPAR α with a K_d in the low micromolar range (13), whereas nafenopin, with a similar potency, does not bind at all (58). Furthermore, only a few PPAR α ligands, such as WY-14643, consistently bind to PPAR α in in vitro assays and also induce PPAR transactivation in gene reporter assays (11, 57). For example, fibrates, such as ciprofibrate and bezafibrate, do not bind to PPAR α in coactivator-dependent binding assays, although they induce PPAR transactivation (57). Thus, it is possible that immediate fibrate-induced metabolic effects, preceding the expression of PPAR α target genes, might contribute to PPAR α sustained activation independently or in addition to PPAR α activation through direct binding.

The first detectable event in isolated rat liver cells exposed to certain PPs, such as ciprofibrate, is the formation of nonmetabolizable acyl-CoA thioesters of the drugs, which attain a steady-state concentration after 30 min, amounting to a significant proportion of total cell CoASH and most of cytosolic CoASH (5, 59). A high concentration of acyl-CoA thioesters of PPs is also observed in the liver of PP-treated rats (6). The resulting immediate sequestration and unavailability of CoASH may result in a transient increased concentration of ω -hydroxylation products, which could contribute to PPAR α activation, because, in contrast to β -oxidation, CoASH is not required for these oxidative pathways. Furthermore, incorporation of EETs to phospholipids also requires previous activation to CoASH esters (46), and CoASH unavailability may direct EET metabolism to ω -hydroxylation and other oxidative pathways. The subsequent induction of β -oxidation enzymes, of CoASH synthesis (7, 8), and of acyl-CoA thioesterases, upon longer PPs exposure (60), probably would contribute to the feedback control of these putative and short-term concentration changes. It is worth noting that HEETs bind to PPAR α with a K_d in the low nanomolar range (i.e., almost 3 orders of magnitude lower than that of PPs with highest PPAR α affinity). Hence, although ligand-activated PPAR α appears essential for peroxisome proliferation, it is possible that low concentrations of other signaling molecules, such as HEETs, but with much greater affinity for PPAR α than ciprofibrate, might be necessary to initiate PPAR α productive binding to response elements in the promoters of target genes.

Although the in vivo involvement of PP-CoA formation in peroxisomal proliferation remains speculative, PPs-CoAs have an essential role in PP-induced hypolipidemic activity. The human liver, which, as already mentioned, is resistant to peroxisomal proliferation, also forms PPs-CoA thioesters (5). There is strong evidence that PP-induced hypolipidemic activity in humans results from the suppression of hepatic nuclear factor-4 α (HNF-4 α) activity by metabolically

formed PPs-CoAs and is PPAR α -independent (61). In the rat, nonproductive PPAR α binding and displacement of HNF-4 α from a response element shared by both transcription factors is thought to be responsible for hypolipidemia (61, 62). HNF-4 α controls genes encoding proteins involved in the production of lipoproteins, such as the apolipoprotein C-III gene (63). The overall effect of HNF-4 α conditional disruption in mouse liver is the reduction of circulating lipid levels (64), consistent with its proposed role as a target of hypolipidemic drugs. Thus, as PP hypolipidemic activity in humans can be explained by a PPAR α -independent mechanism, the main difference with the rat is the inability of human PPAR α to support peroxisomal proliferation, an observation that is ascribed to low PPAR α concentration in human liver (65). However, human PPAR α supports the induction of peroxisomal proliferation in PPAR α -deficient mouse liver (66), and PPAR α concentration does not appear to be limiting for the transcription of PPAR α target genes in the mouse, because they are induced even under reduced PPAR α expression (64).

On this basis, it is tempting to speculate that the liver's capacity to synthesize endogenous PPAR α ligands might be one of the factors responsible for the differences in species sensitivity to peroxisomal proliferation. In mammals, the nature and distribution of P450 isoforms is species-specific and may also depend on age, sex, hormonal status, diet, and the organ (51 and references cited therein). In human liver, EETs are products primarily of *CYP2C8* epoxidase (67, 68). A single human *CYP4A11* has been cloned and shown to catalyze AA and lauric acid $\omega/\omega-1$ hydroxylation (21); however, it is unknown whether this enzyme catalyzes the $\omega/\omega-1$ hydroxylation of EETs. In fact, in vitro studies suggest that *CYP4F3*s are the main catalyst in the oxidation of fatty acid epoxides in humans, and the human *CYP4F* family appears to play a similar functional role as the *CYP4A* family in rats (69). However, little is known about *CYP4F3* in vivo function, and it remains to be established whether differences in P450 monooxygenase specificity or activity might account for the apparent inability of human PPAR α to support peroxisomal proliferation.

In summary, this is the first report showing that *CYP2C* and *CYP4A* are involved in ciprofibrate-induced peroxisomal proliferation in isolated rat liver cells. It suggests that metabolites of these P450 isoforms might contribute to or be responsible for PPAR α -dependent peroxisomal proliferation, representing a putative alternative mechanism to the canonical view of PP-induced PPAR α activation. ■

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