0026-895X/00/030470-00$3.00/0
MOLECULAR PHARMACOLOGY
Vol. 58, No. 3
63/849110
Mol Pharmacol 58:470–476, 2000
Printed in U.S.A.

Evidence for Peroxisome Proliferator-Activated Receptor (PPAR)α-Independent Peroxisome Proliferation: Effects of PPARγ/δ-Specific Agonists in PPARα-Null Mice

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Received February 2, 2000; accepted June 15, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

Peroxisome proliferators are a diverse group of compounds that cause hepatic hypertrophy and hyperplasia, increase peroxisome number, and on chronic high-dose administration, lead to rodent liver tumorigenesis. Various lines of evidence have led to the conclusion that these agents induce their pleiotropic effects exclusively via agonism of peroxisome proliferator-activated receptor (PPAR)α, a member of the steroid receptor superfamily involved in the regulation of fatty acid metabolism. Recently, agonists of two other members of this receptor family have been identified. PPARγ is predominantly expressed in adipocytes where it mediates differentiation; PPARδ is a widely expressed orphan receptor with yet unresolved physiologic functions. In the course of characterizing newer PPAR ligands, we noted that highly selective PPARγ agonists or dual PPARγ/PPARδ agonists, lacking apparent murine PPARα agonist activity, cause peroxisome proliferation in CD-1 mice. We therefore made use of PPARα knockout mice to investigate whether these effects resulted from agonism of PPARα by these agents at very high dose levels or whether PPARγ (or PPARδ) agonism alone can result in peroxisome proliferation. We report here that several parameters linked to the hepatic peroxisome proliferation response in mice that were seen with these agents resulted from PPARα-independent effects.

There are three related members of the peroxisome proliferator-activated receptor (PPAR) family—PPARα, PPARγ, and PPARδ—that are subjected to regulation by fatty acids and lipid metabolites (Schoonjans et al., 1996). Individual PPARs dimerize with the retinoid X receptor and the PPAR-retinoid X receptor complex binds to specific DNA response elements [peroxisome proliferator response elements (PPREs) composed of hexanucleotide direct repeats] in gene promoters and functions as a ligand-activated transcription factor (Gearing et al., 1993). After ligand binding, the PPAR ligand binding domain undergoes specific conformational changes allowing for the recruitment of one or more coactivator proteins (such as steroid receptor coactivator 1 or cAMP-response element-binding protein). The receptor-coactivator complex then interacts with components of the basal transcriptional apparatus, resulting in the induction of RNA transcription (McInerney et al., 1998).

PPARα is expressed in liver and other tissues including kidney, heart, and to a lesser degree, muscle and brown fat. Activation of PPARα in liver of mice or rats stimulates fatty acid oxidation and peroxisome proliferation (PP). Known PPARα target genes include the enzymes of peroxisomal and mitochondrial fatty acid β-oxidation, liver fatty acid-binding protein *lfabp*, and carnitine palmitoyl transferase (Kaikaus et al., 1993). PPARγ is expressed at highest levels in adipose tissue where it mediates transcriptional activation of the promoters for *aP2* (adipocyte fatty acid-binding protein) and other adipocyte genes involved in regulation of lipid uptake and lipogenesis (Schoonjans et al., 1996). PPARδ is widely expressed; however, its target genes and physiologic effects are not known.

Compounds that cause PP in rodents are generally believed to act exclusively through the PPARα receptor because the potent and selective mPPARα agonist WY14643 is ineffective in inducing PP in PPARα-null mice (Lee et al., 1995; Gonzalez et al., 1998). We have observed, however, that selected compounds that are specific PPARγ agonists, such as some of the insulin-sensitizing thiazolidinediones (TZDs)

ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; PP, peroxisome proliferation; TZD, thiazolidinedione; FFA, free fatty acid; WT, wild-type; PPRE, peroxisome proliferator response element; CoA, coenzyme A; ACO, acyl-CoA-oxidase; SSPE, saline/sodium phosphate/EDTA; FABP, fatty acid-binding protein; *lfabp*, liver FABP gene; MOPS, 4-morpholinepropanesulfonic acid.

(Lehmann et al., 1995; Berger et al., 1996; Elbrecht et al., 1996), can cause PP in mice when administered at high dose levels. There are at least four potential mechanisms that might account for this observation: 1) compounds that lack significant murine PPARα activity based on in vitro assays may possess weak PPARα activity in vivo; 2) such compounds may undergo in vivo metabolism, resulting in de novo generation of PPARα agonists; 3) high occupancy of PPARγ in liver may be sufficient to mediate some effects normally attributed to PPARα; 4) compounds exhibiting these effects might act through another related receptor, such as PPARδ, which is expressed in the liver and could function as a surrogate for PPARα. Because it would be desirable to develop therapeutic agents lacking the liability of rodent PP and the associated risk of tumorigenesis, the question arises of whether it would be possible to eliminate PP potential by increasing the PPARγ specificity of compound candidates. We report here the results of studies with PPARα-null mice investigating whether PPARγ- or -γ/δ-specific agonists are capable of inducing PP in the absence of PPARα.

Materials and Methods

Compounds. Compounds used in this study include a potent known PPARγ-specific TZD agonist {5-(4-[2-[methyl-(2-pyridyl)amino]ethoxy]benzyl)thiazolidine-2,4-dione}, previously described in (Berger et al., 1996). A non-TZD compound with potent murine PPARγ and PPARδ (but not PPARα) agonist activity, L-783,483 (Berger et al., 1999), provided by Dominick F. Gratale (Merck Research Laboratories, Rahway, NJ), was also used. A known PPARα selective agonist (Kliewer et al., 1994), WY14643 {[4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid}, was purchased from Chemsyn Science Laboratory (Lenexa, KS).

Animals and Assessment of In Vivo Parameters. CD-1 (ICR) BR mice (approximately 5 weeks of age at study initiation; Charles River, Raleigh, NC) or homozygous PPARα-null or sex- and age-matched wild-type (WT) Sv/129 mice (approximately 9 weeks of age at study initiation) (Lee et al., 1995) were allowed ad libitum access to rodent chow (Purina #5001; Purina Mills Inc., Brentwood, MO) and water. Animals were dosed daily by gavage with vehicle (0.5% carboxymethylcellulose) with or without PPAR agonists at the indicated doses. After treatment for 4 or 6 days, the animals were euthanized by isofluorane asphyxiation (CD-1) or pneumothorax under Nembutal anesthesia. Livers were rapidly removed and weighed, and samples were placed in ice-cold phosphate buffer for immediate processing or in liquid nitrogen and stored at −80°C for subsequent preparation of total RNA or measurement of enzyme levels or activity. Additional samples were taken for light and electron microscopy. Plasma glucose, triglyceride, and free fatty acid (FFA) concentrations were determined from blood obtained at necropsy. Glucose and triglyceride determinations were performed using standard glucose oxidase (Sigma, St. Louis, MO) and glycerol kinase (Boehringer Mannheim Biochemica, Indianapolis, IN) assays, respectively. FFA levels were determined by the acyl-coenzyme A (CoA) synthetase/acyl-CoA oxidase/peroxidase method (Boehringer Mannheim Biochemica). All in vivo experiments were approved by the Institutional Animal Care and Use Committee.

Measurement of Hepatic Acyl-CoA-Oxidase (ACO) and CYP4A Levels. Hepatic ACO activity was measured spectrophotometrically using leuco-dichlorofluorescein as the chromophore (Walusimbi-Kisitu and Harrison, 1983). Briefly, 20 μl of whole liver homogenate, diluted 1:40 with 12 mM sodium/potassium phosphate buffer, pH 7.4 (dilutions were increased as necessary to keep the rate of increase in $A < 0.08$ absorbance units/min) was incubated with 0.02% Triton X-100, 40 mM aminotriazole, 2.3 U of horseradish peroxidase, 0.6 μM leuco-dichlorofluorescein, and 33 μM palmitoyl-CoA in a 30°C cuvette. The increase in A at 502 nm was monitored for ~1.5 min after a lag period of 2 min against a blank lacking palmitoyl-CoA in a final volume of 0.92 ml.

CYP4A protein levels were assessed in whole liver homogenate, and samples were solubilized in 0.5% sodium cholate/Triton X-100, using rabbit anti-rat CYP4A1 as the primary antibody (Gentest Corp., Waltham, MA) and goat anti-rabbit Ig, horseradish peroxidase-conjugated, as the secondary antibody (Pierce Chemical Co., Rockford, IL). Solubilized samples were diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6, to give approximately 20 ng of protein/50 μl. This was adsorbed to 96-well Maxisorp titer plates (Nalge Nunc International, Rochester, NY) for 1 h at 37°C. Wells were then rinsed four times with 0.05% Tween 20 in Ca^{2+}- and Mg^{2+}-free PBS (rinse/block buffer) and 100 μl of primary antibody diluted 1:4000 in Superblock (Pierce Chemical Co.) containing 0.05% Tween 20 (antibody dilution buffer) was added to each well. After 1 h of incubation at 37°C, plates were rinsed as above with rinse/block buffer, 100 μl of secondary antibody diluted 1:2000 in antibody dilution buffer was added, and plates were incubated for 1 h at 37°C. After incubation, plates were rinsed as above, 100 μl of ABTS 1 STEP (Pierce Chemical Co.) was added, and plates were incubated at room temperature for 20 min. The reaction was stopped by addition of 50°C 2% SDS containing 0.5 mg/ml Proteinase K, and plates were read immediately in a SpectraMax 340 plate reader (Molecular Devices, Sunnyvale, CA).

Measurement of PPARα Target Gene Expression. Frozen tissues were pulverized and then homogenized in 10 volumes of ULTRA SPEC buffer (Biotecx Laboratories, Inc. Houston, TX) according to the manufacturer's specifications, followed by extraction of total RNA as described previously (Chomczynski and Sacchi, 1987). Aliquots of total RNA (15 μg) were denatured in a solution of 2.2 M formaldehyde and 50% formamide, 1× MOPS/EDTA buffer (Digene Diagnostics, Inc., Beltsville, MD), and 0.01 mg/ml ethidium bromide by heating at 70°C for 10 min. The samples were cooled, and gel loading buffer (0.5% xylene cyanol, 0.5% bromophenol blue, 40% sucrose, 2.2 M formaldehyde, and 50% formamide) was added. The samples were then loaded onto 1.2% SeaKem Gold agarose (FMC BioProducts, Rockland, ME) gels in 1× MOPS. After electrophoresis, RNA was transferred to Duralon-UV membranes (Stratagene, La Jolla, CA) overnight in 20× saline/sodium phosphate/EDTA (SSPE) (Digene Diagnostics, Inc.) (Ausubel et al., 1987). After UV crosslinking with UV Crosslinker 1800 (Stratagene), Northern blots were prehybridized in Express-Hyb (CLONTECH Laboratories, Inc., Palo Alto, CA) at 60°C for 1 h then hybridized to specific ^{32}P-labeled cDNA probes at a concentration of 1 to 4×10^6 cpm/ml. The hybridization was carried out for at least 16 h at 60°C. The blots were washed twice in 2× SSPE, 0.1% SDS at 60°C for 30 min each and then twice in 0.1× SSPE, 0.1% SDS at 50°C for 10 min. The membranes were sealed in plastic and exposed to a PhosphorImager screen. The screens were analyzed on a Molecular Dynamics PhosphorImager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

For probe generation, first-strand cDNA synthesis was achieved using reverse transcriptase and other reagents from Boehringer Mannheim. A 396-base pair human ACO fragment was amplified from HepG2 cell total RNA using the following primer pair: 5′-ATGAACCCGGACCTGCGCAG-3′/5′-TGATCTCCAAGTTCCAGGCGG-3′ (coding/noncoding strand). Likewise, 390-bp rat liver *lfabp* fragment was amplified using the following primer pair: 5′-ATGAACTTCTCCGGCAAGTACC-3′/5′-CCTTGTCTAAATTCTCTTGC-3′ (coding/noncoding strand). The primer sequences were based on published sequences. The polymerase chain reaction products, as well as DNA fragments containing the mouse PPARγ and PPARδ ligand binding domains, were gel purified and used as templates for random prime labeling (Life Technologies, Gaithersburg, MD). Northern blots were stripped and rehybridized with a 484-base pair 23-kDa highly basic protein cDNA generated from a control amplimer set from CLONTECH Laboratories, Inc. to control for small differences in RNA loading and transferring.

Microscopic Examination. At necropsy, tissue samples were placed in 10% neutral buffered Formalin. Tissues were processed by routine methods and embedded in paraffin. Sections of liver, approximately 5 μm thick, were immunohistochemically stained with a rabbit polyclonal antibody to rat and mouse PMP70 (Affinity Bioreagents, Golden, CO), a peroxisomal membrane protein known to be induced by peroxisome proliferators (Baumgart et al., 1989). For transmission electron microscopy, liver sections were fixed in 4% formaldehyde + 1% glutaraldehyde solution, and 1 mm^3 blocks were cut, washed in 0.15 M phosphate buffer, postfixed in a 1% osmium tetroxide solution, and embedded in Epon 812 (Polysciences Inc., Warrington, PA) after alcoholic dehydration. Blocks of liver from all control and treated animals were cut using a Reichert Ultracut ultramicrotome (Leica, Deerfield, IL). Toluidine blue-stained semithin sections were examined by light microscopy to assess the adequacy of tissue preservation for ultrastructural examination and to select similar centrilobular and periportal areas in the liver of control and treated animals. Uranyl acetate- and lead citrate-contrasted ultrathin sections were then examined using a CM12 (Philips/FEI Co., Hillsboro, OR) transmission electron microscope at 80 keV. From each animal, micrographs of hepatocytes were randomly obtained during microscopic observation of the liver for examination of the peroxisome content. The number of hepatic peroxisomes (differentiated from mitochondria based on ultrastructure) was semiquantitatively assessed (from + = few to ++ = numerous) for each group, based on the observation of approximately 20 micrographs per group.

Results and Discussion

PP Can Be Caused by Compounds Lacking In Vitro PPARα Activity. Using cloned isoforms of PPARγ, PPARδ, and PPARα, we previously established (Berger et al., 1996) that the TZD compound used in this study was a potent ($K_i <$ 50 nM) PPARγ agonist without detectable PPARδ or PPARα activity (up to 30 μM in in vitro assays). L-783,483 was also previously shown (Berger et al., 1999) to lack murine PPARα activity, although it has potent activity on both murine PPARγ (29 nM) and PPARδ (30 nM). Finally, the potent rodent PPARα agonist WY14643 was shown not to have detectable in vitro PPARγ or PPARδ agonist activity (data not shown). In studies to determine whether compounds with PPARγ (and PPARδ) activity might be able to induce hepatic PP, liver weight, ACO activity, and CYP4A levels were measured after four daily doses (500 mg/kg/day) of TZD or L-783,483 were administered to CD-1 mice (n = 4, each sex). The results of these studies are shown in Table 1. At these dose levels, both compounds were effective in inducing increases in all three parameters. Treatment with the TZD compound resulted in increases in mean liver weight of 17 to 27% versus 36 to 59% increases with L-783,483. Mean hepatic ACO activity was increased by 245 to 260% with TZD and by 460 to 720% with L-783,483 treatment; in addition, mean CYP4A levels were increased by 125 to 221% with TZD and by 206 to 466% with L-783,483. Thus, in vivo treatment of normal mice with a PPARγ-selective agonist or a dual PPARγ/PPARδ agonist resulted in substantial hepatic effects that were consistent with induction of the PP pathway. With the possible exception of the observations of Kolattukudy et al. of PP in the uropygial glands of mallard ducks in response to estradiol (Bohnet et al., 1991; Ma et al., 1998), this represents the first demonstration of induction of hepatic ACO or CYP4A enzymes by compounds with PPARγ and/or PPARδ activity.

Effects of PPAR Agonists in PPARα-Null Mice. In vivo experiments were conducted using male PPARα-null mice and matched controls (WT) to assess whether the effects seen in CD-1 mice were mediated by spillover onto PPARα or by independent mechanisms (e.g., occupancy and activation of hepatic PPARγ and/or PPARδ mimicking the effect of PPARα activation). In an initial study, both PPARα WT and PPARα-null mice (n = 4 males in each group) were treated with four daily doses of vehicle, TZD, or L-783,483 just as CD-1 mice had been. There was clear evidence of PP in the PPARα WT mice; in the null mice, the results were suggestive of a modest degree of induction—in particular, both TZD and L-783,483 appeared to retain the capacity to induce ACO activity (data not shown). To firmly establish that induction of parameters related to PP could occur in the absence of PPARα, a second study was performed in which groups of four male mice were treated with six daily doses of TZD or L-783,483 (500 mg/kg/day, each). A separate group of mice also received a PPARα-specific agonist, WY14643 (50 mg/kg/day); we have also verified that this compound is a potent (75 nM) and specific (no PPARγ or PPARδ activity) PPARα agonist using cloned murine isoforms (Berger et al., 1999).

Effects on Organ Weights and Clinical Parameters. Table 2 shows the effects of these compounds on liver weight and blood levels of glucose, triglycerides, and FFAs. In PPARα WT animals, all three compounds caused significant decreases in serum triglyceride levels. In the null mice, however, no decrease in triglycerides was seen with any of the compounds. The effect of fibrates and compounds such as WY14643 to lower triglyceride levels has been proven to be mediated by PPARα (Peters et al., 1997). Our results, therefore, suggested that triglyceride lowering was mediated al-

TABLE 1

Liver weight, ACO, and CYP4A in CD-1 mice

Four CD-1 mice (each sex) were treated for 4 days as indicated; data for TZD represent two independent studies (eight animals per sex total). Values in parentheses represent percentage increase over concurrent vehicle control. CYP4A values have had an average preimmune background of 0.16 AU subtracted (previously determined).

	Liver Weight ± S.E.M.		ACO ± S.E.M.		CYP4A ± S.E.M.	
	Male	Female	Male	Female	Male	Female
	% body weight (% change)		*nmol/min/mg (% change)*		*AU (% change)*	
Vehicle	6.19 ± 0.16	5.22 ± 0.14	3.16 ± 0.21	3.69 ± 0.29	0.33 ± 0.04	0.26 ± 0.04
TZD, 500 mg/kg/day	7.25 ± 0.26 (17)**	6.65 ± 0.11 (27)**	11.38 ± 2.98 (260)*	12.73 ± 2.86 (245)*	0.73 ± 0.05 (125)**	0.84 ± 0.05 (221)**
Vehicle	5.63 ± 0.11	5.20 ± 0.13	3.48 ± 0.42	4.63 ± 0.67	0.31 ± 0.05	0.19 ± 0.02
L-783,483, 500 mg/kg/day	7.65 ± 0.23 (36)**	8.27 ± 0.23 (59)**	24.63 ± 3.21 (720)**	25.95 ± 3.37 (460)*	0.94 ± 0.06 (206)**	1.06 ± 0.13 (466)*

* $P \leq .05$; ** $P \leq .01$. P values were calculated using the appropriate t test (equal or unequal variance) based on F test results.

TABLE 2
Effect of PPAR agonist treatment on in vivo parameters and liver weight in WT versus PPARα-null mice
Mice were treated as indicated for 6 days. All parameters are expressed as mean values (±S.E.).

Treatment	Glucose	Triglyceride	FFA	Liver Weight
	mg/dl		*mmol/l*	*% body weight (% change)*
WT mice				
Vehicle	131 ± 7	78.5 ± 12	0.60 ± 0.06	3.65 ± 0.16
WY14643, 50 mg/kg/day	117 ± 11	33.4 ± 9*	0.49 ± 0.03	5.86 ± 0.23** (60.5)
TZD, 500 mg/kg/day	126 ± 10	26.7 ± 5**	0.08 ± 0.02**	4.94 ± 0.19** (35.2)
L-783,483, 500 mg/kg/day	111 ± 17	18.9 ± 3**	0.18 ± 0.15*	6.04 ± 0.32** (65.5)
PPARα-null mice				
Vehicle	123 ± 6	95.7 ± 21	0.71 ± 0.06	4.39 ± 0.39
WY14643, 50 mg/kg/day	129 ± 8	74.1 ± 7	0.68 ± 0.13	4.13 ± 0.22 (−6)
TZD, 500 mg/kg/day	125 ± 7	77.0 ± 5	0.37 ± 0.03**	5.66 ± 0.15* (28.8)
L-783,483, 500 mg/kg/day	98 ± 10	73.3 ± 19	0.25 ± 0.09**	6.97 ± 0.97* (58.6)

* $P < .05$; ** $P \leq .01$. P values were calculated using a t test assuming equal variance.

most exclusively by PPARα with little contribution from PPARγ (or PPARδ) and, further, that some degree of PPARα activation was being effected by the PPARγ/δ-specific compounds in the intact animal, in contrast with the results seen in vitro with cloned receptors. In accord with the known physiologic effects of PPARγ activation on lipid metabolism (Schoonjans et al., 1997), FFA levels were significantly lowered by both compounds with PPARγ activity (in both WT and null mice) but not by WY14643. PPARγ agonists are effective in lowering glucose in insulin-resistant hyperglycemic states but do not elicit hypoglycemia; thus, as expected, glucose levels were not lowered in any treatment group below the normal levels present in both WT or null mice.

Hepatic weight increases, one of the hallmarks of PP (as well as other forms of liver enzyme induction), were clearly elicited by all three compounds in WT mice. However, in PPARα-null mice, treatment with the PPARα agonist WY14643 failed to induce liver weight increases, but both TZD and L-783,483 still caused substantial increases in liver weight. Therefore, one PPARα-associated effect, liver weight increases, but not another, triglyceride lowering, was apparently mediated by either PPARδ or PPARγ.

Induction of PPARα Target Genes. Table 3 shows the effect of treatment on ACO activity and CYP4A content, two widely used markers of PP. PPARα WT animals displayed highly significant increases in both parameters in response to treatment with all three compounds. Although the extent of CYP4A induction was similar among compounds, WY14643 and L-783,483 caused larger increases in ACO activity (~18× and ~15×, respectively) versus only a modest increase of ~4.5× with TZD. In PPARα-null animals, WY14643, as expected, had no effect on either ACO activity or CYP4A content. In contrast, treatment with either TZD or L-783,483 increased both parameters in PPARα-null mice. The increases in ACO activity were modest relative to WT animals, but the induced levels of CYP4A were equivalent to WT for L-783,483 and approximately half that seen in WT for TZD (percentage increases were actually greater than those in WT mice due to low basal levels CYP4A).

To corroborate and extend the enzymatic data, mRNA expression levels of ACO, as well as another PPARα target gene, liver FABP, were also determined by Northern blot analysis. As depicted in Fig. 1, A to E, *lfabp* gene expression in liver from WT mice was induced (by ≈2- to 3-fold) with all three drug treatments. Similarly, each treatment resulted in substantial (3- to 4-fold) induction of ACO mRNA levels in liver from WT mice. In PPARα-null mice, residual induction of *lfabp* expression was evident with TZD and L-783,483 treatment. Importantly, induction of liver ACO mRNA expression was also readily observed in liver from null mice treated with TZD or L-783,483; 2.4-fold induction occurred after TZD treatment, and 2.7-fold induction occurred after L-783,483 treatment. As expected, WY14643 failed to stimulate ACO mRNA expression in null mice.

ACO, CYP4A, and liver FABP are classical hepatic target genes that respond to PPARα activation as part of the PP response. All three genes are known to contain functionally intact PPREs in their promoters. The above results indicate that compounds possessing PPARγ and/or PPARδ agonist activity are capable of inducing each of these known target genes in livers of both WT and PPARα-null mice. Thus, although hepatic expression levels of PPARγ and PPARδ are thought to be substantially lower than PPARα (in mice and rats, but see below), it appears that residual PPARγ or

TABLE 3
Effect of PPAR agonist treatment on ACO activity and CYP4A content in WT versus PPARα-null mice
Mice were treated as indicated for 6 days. All parameters are expressed as mean values (±S.E.). CYP4A values have had an average "no sample" background of 0.10 AU subtracted.

Treatment	PPARα WT		PPARα-Null	
	ACO Activity	CYP4A Content	ACO Activity	CYP4A Content
	nmol/min/mg (% change)	*AU units (% change)*	*nmol/min/mg (% change)*	*AU units (% change)*
Vehicle	2.38 ± 0.19	0.16 ± 0.03	2.69 ± 0.35	0.077 ± 0.013
WY14643, 50 mg/kg/day	43.11 ± 4.57** (1713)	0.43 ± 0.01** (165)	2.65 ± 0.07 (−2)	0.082 ± 0.006 (6)
TZD, 500 mg/kg/day	10.64 ± 1.34** (348)	0.45 ± 0.04** (180)	6.80 ± 0.48** (153)	0.26 ± 0.02** (238)
L-783,483, 500 mg/kg/day	35.04 ± 2.53** (1374)	0.49 ± 0.02** (202)	9.90 ± 1.32** (268)	0.45 ± 0.04** (481)

* $P \leq .05$; ** $P \leq .01$. P values were calculated using the appropriate t test (equal or unequal variance) based on F test results.

PPARδ receptors expressed in liver (or possibly other yet-to-be determined orphan receptors) are sufficiently promiscuous that they may, at the suprapharmacologic doses of the potent agonists tested here, be capable of mimicking PPARα function.

It should be noted that Brun et al. (1996) have seen a degree of cross-activation between PPARγ and PPARα with respect to the transcription of adipocyte differentiation genes. In addition, Edvardsson et al. (1999) have recently observed indications of PP in the livers of ob/ob mice treated

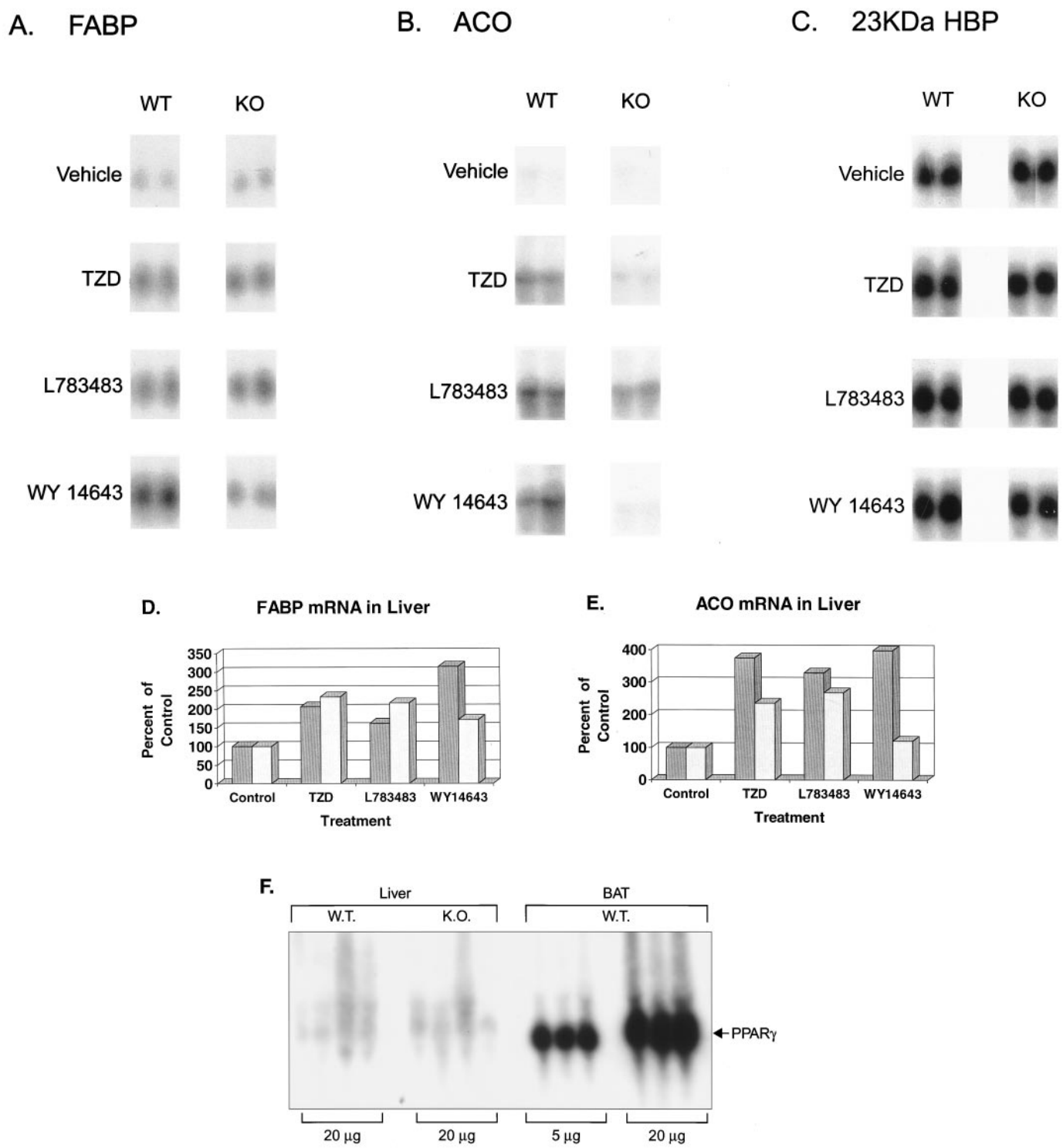

Fig. 1. mRNA expression levels. mRNA was isolated from the livers of WT or PPARα-null mice treated as indicated. A and B, duplicate lanes of Northern blots probed with cDNAs corresponding to two PPARα target genes, liver FABP (A) and ACO (B). C, mRNA expression levels of a gene not containing a PPRE, 23-kDa highly basic protein (HBP), used to normalize for RNA loading and transfer differences. D and E, quantitation of mean mRNA expression levels for liver FABP (D) or ACO (E) after normalizing against minor variation in 23-kDa highly basic protein expression. Shaded columns, WT; open columns, knockout (KO). F, expression of PPARγ in the livers of WT and PPARα-null mice compared with levels in brown adipose tissue (BAT).

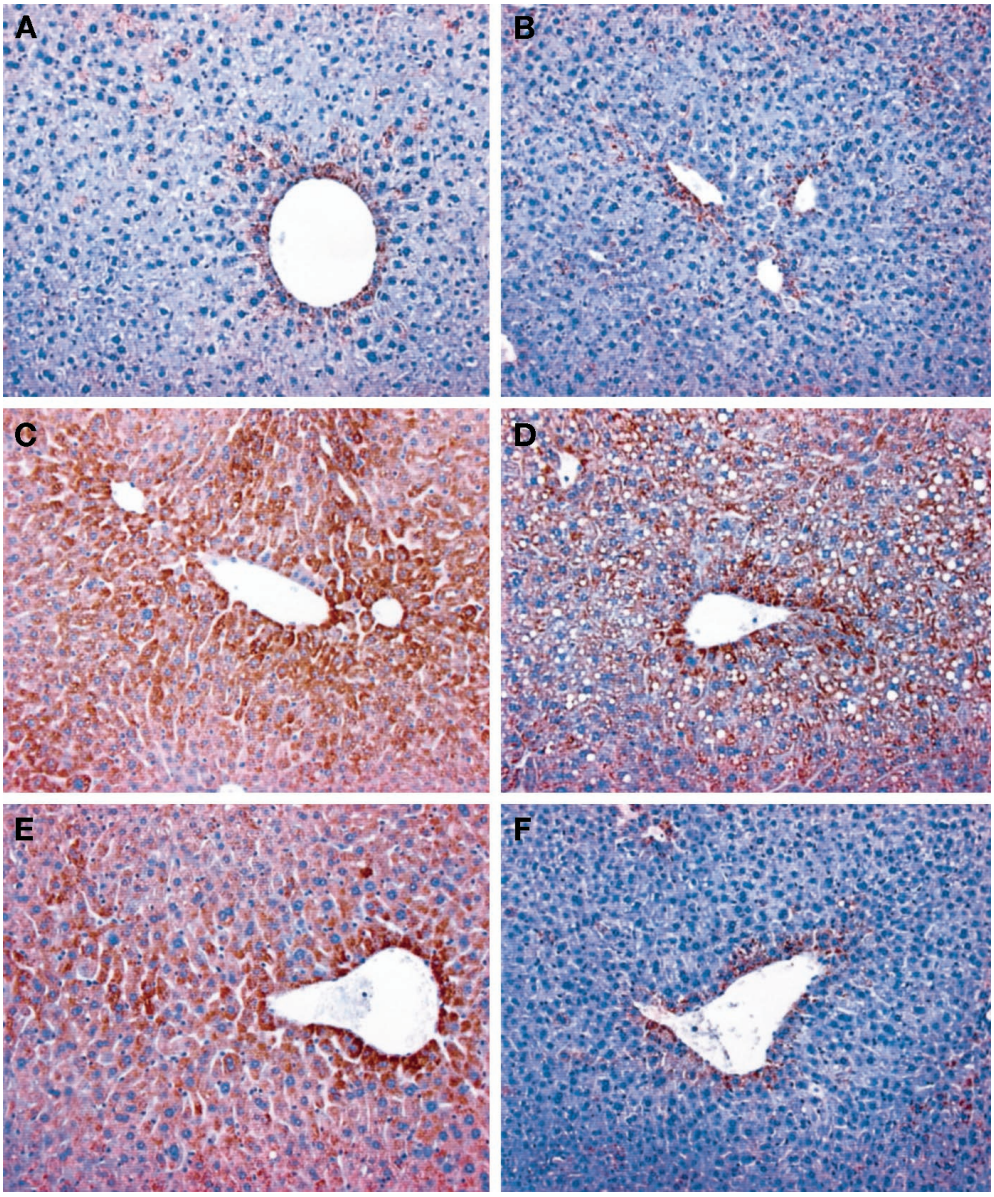

Fig. 2. Light micrographs of liver sections. Sections of liver from WT and PPARα-null mice were immunohistochemically stained for the peroxisomal membrane protein PMP70 and examined by light microscopy. A (WT) and B (PPARα-null), vehicle-treated mice; C (WT) and D (PPARα-null), L-783,483-treated mice; E (WT) and F (PPARα-null), WY14643-treated mice.

with a PPARγ agonist but not in their lean littermates (much lower doses of agonist were used in these studies, <1 mg/kg/day). They attributed this difference to elevated expression of PPARγ2 in the livers of the ob/ob mice. In this regard, it has been reported by Costet et al. (1998) that fat-filled hepatocytes from aging PPARα-null mice have elevated expression levels of PPARγ2. To investigate the possibility that the PPARα-null mice used in the studies reported here may have had elevated hepatic PPARγ expression levels, which could have contributed to the PP effects we saw, we examined the expression levels of PPARγ mRNA in the livers of both WT and null mice (Fig. 1F). Also shown are blots of brown adipose tissue from WT mice. It can be seen that there is very little PPARγ expression in the livers of either WT or PPARα-null mice and that there is no discernable difference between them. The same was true for PPARδ (data not shown).

TABLE 4
Semiquantitative assessment of hepatic peroxisome content
Mice were treated as indicated for 6 days.

Mice	Treatment			
	Control	TZD 500 mg/kg/day	L-783,483 500 mg/kg/day	WY14643 50 mg/kg/day
WT	+/−	+	++	++
Null	+/−	+	++	+/−

+/−, few peroxisomes; +, some peroxisomes; ++, numerous peroxisomes.

Effect of PPAR Agonists on Liver Histomorphology. Ultimately, hepatic PP is defined as an increase in the peroxisomal content of hepatocytes. Consequently, liver sections from each of the treatment groups were processed for both light and electron microscopy. The sections processed for light microscopy were stained with antisera to a peroxisomal membrane protein, PMP70. Examples of control, L-783,483-, and WY14643-treated liver sections are shown in Fig. 2. Significant induction was seen in both L-783,483- and WY14643-treated WT mice, but only L-783438 (and, to a lesser extent, TZD; data not shown) caused induction in null mice. The sections processed for transmission electron microscopy were used both to confirm that the changes seen in PMP70 were reflected in an actual increase in ultrastructur-

ally identifiable peroxisomes as well as being subjected to semiquantitative analysis, as shown in Table 4. It can be seen that, on the basis of ultrastructural identification, the increase in PMP70 caused by L-783,483 in null mice does correlate with an increase in the number of peroxisomes. In addition, the lesser degree of PP caused by TZD, as determined from the electron micrographs (Table 4) also correlated with the level of PMP70 expression (data not shown). Although the zonal gradation of staining does not appear as clear-cut in the null mice as it is in the WT mice, this is likely due to the obscuring effect of the numerous vacuoles. Specific staining indicated that these vacuoles were lipid-filled and probably are indicative of an inability of the null mice to maintain lipid homeostasis in the face of metabolic stress produced by the administration of the PPARγ/δ agonist.

In conclusion, we have demonstrated, for the first time in mice, that in vivo exposure to compounds with PPARγ and PPARδ activity that lack in vitro PPARα activity is sufficient to induce PP. Moreover, we have shown that several parameters of PP can be induced by such compounds in mice lacking PPARα receptors. We hypothesize that this is the result of functional overlap that exists between members of the PPAR subfamily of nuclear receptors. This conclusion has important implications for the future development of therapeutic agents targeted as "selective" agonists of individual PPAR isoforms.

Acknowledgments

We are indebted to Dominick F. Gratale, Gerard Kieczykowski, Philip Eskola, Joseph F. Leone, and Peter A. Cicala (Merck Research Laboratories, Rahway, NJ) and to Gary Dysart, John Frank, Katy McGettigan, and Gordon Wollenberg (Merck Research Laboratories, West Point, PA) for technical assistance.

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