Diminished Hepatocellular Proliferation in Mice Humanized for the Nuclear Receptor Peroxisome Proliferator-Activated Receptor α

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ABSTRACT

Lipid-lowering fibrate drugs function as agonists for the nuclear receptor peroxisome proliferator-activated receptor α (PPARα). Sustained activation of PPARα leads to the development of liver tumors in rats and mice. However, humans appear to be resistant to the induction of peroxisome proliferation and the development of liver cancer by fibrate drugs. The molecular basis of this species difference is not known. To examine the mechanism determining species differences in peroxisome proliferator response between mice and humans, a PPARα-humanized mouse line was generated in which the human PPARα was expressed in liver under control of the tetracycline responsive regulatory system. The PPARα-humanized and wild-type mice responded to treatment with the potent PPARα ligand Wy-14643 as revealed by induction of genes encoding peroxisomal and mitochondrial fatty acid metabolizing enzymes and resultant decrease of serum triglycerides. However, surprisingly, only the wild-type mice and not the PPARα-humanized mice exhibited hepatocellular proliferation as revealed by elevation of cell cycle control genes, increased incorporation of 5-bromo-2′-deoxyuridine into hepatocyte nuclei, and hepatomegaly. These studies establish that following ligand activation, the PPARα-mediated pathways controlling lipid metabolism are independent from those controlling the cell proliferation pathways. These findings also suggest that structural differences between human and mouse PPARα are responsible for the differential susceptibility to the development of hepatocarcinomas observed after treatment with fibrates. The PPARα-humanized mice should serve as models for use in drug development and human risk assessment and to determine the mechanism of hepatocarcinogenesis of peroxisome proliferators.

INTRODUCTION

Peroxisome proliferators are a structurally diverse group of chemicals including naturally occurring steroids and lipids and the commonly prescribed hypolipidemic fibrate drugs (used to treat dyslipidemias), pesticides, industrial plasticizers, and solvents. They exert their effects by activating peroxisome proliferator-activated receptor α (PPARα). Treatment with peroxisome proliferators results in a short-term pleiotropic response that is manifest by liver hyperplasia and hypertrophy, proliferation of peroxisomes, and increases in oxidation of fatty acids through induction of genes encoding mitochondrial, peroxisomal, and microsomal fatty acid oxidation systems (1, 2). Peroxisome proliferators have been shown to act as nongenotoxic carcinogens; long-term administration to rats and mice results in the formation of hepatocellular carcinomas; however, humans appear to be resistant to the induction of peroxisome proliferation and the development of hepatocarcinomas by such chemicals (3).

Treatment with peroxisome proliferators significantly increases levels of peroxisomal fatty acid β-oxidation system, including acetyl-CoA oxidase and the CYP4A subfamily of enzymes (microsomal ω-oxidation) that leads to the generation of hydrogen peroxide (H₂O₂) (4). Oxidative stress and production of reactive oxygen species caused by sustained overproduction of H₂O₂ and the resulting DNA damage (caused by disproportionate increases in H₂O₂-generating oxidses and H₂O₂-degrading enzyme catalase contained within the peroxisome) and liver cell proliferation contribute to liver tumor development in rodents (5). Whereas high rates of hepatocyte proliferation have been correlated with increased risk for development of hepatocellular carcinomas, the development of tumors also requires DNA damage in concert with cell proliferation to fix the damage into gene mutations. Increased expression of cyclins, cyclin-dependent kinases (CDKs), proliferating cellular nuclear antigen (PCNA), and c-myc has been used as biomarkers of increased cell proliferation, although whether an increase in cyclin or CDK expression is a cause or an effect of carcinogenesis has not been elucidated (6–9). A strong correlation between high levels of peroxisome proliferation (and H₂O₂-generating acyl-CoA oxidase) and liver carcinogenesis has been established for the peroxisome proliferator di(2-ethylhexyl)phthalate in rodents (10).

Targeted disruption of the mouse PPARα gene has confirmed that this receptor is responsible for peroxisome proliferator-induced pleiotropic responses in mice, including the development of hepatocarcinomas (11, 12). The mechanism of species difference in response to peroxisome proliferators is unknown but may be related to differences in the expression and activity of PPARα between susceptible species (rats and mice) and humans (13). Human PPARα has been shown to be functional in transactivation assays, although some differences in the affinity of ligands for the human and mouse receptor have been observed (14, 15). In humans, decreased expression levels of PPARα or the presence of splice variants was suggested to contribute to the resistance of humans to peroxisome proliferation on treatment with fibrate drugs (16, 17). In this regard, forced expression of human PPARα in HepG2 cells and transient retroviral overexpression of the human receptor in mice resulted in induction of some PPARα target genes, indicating that human PPARα target genes are responsive and that human PPARα is a functional receptor (18–20).

This study describes the generation of PPARα-humanized mice that express human PPARα in a mouse PPARα null background. When treated with peroxisome proliferators, these mice exhibit decreased serum triglycerides and marked increases in genes encoding peroxisomal, mitochondrial, and microsomal fatty acid oxidation enzymes, albeit to a lesser extent than wild-type mice. Strikingly, unlike wild-type mice, the PPARα-humanized mice do not display increases in Wy-14,643-induced replicative DNA synthesis or increased expression of cell cycle control genes in the liver. The data indicate that the difference in carcinogenic responses observed after treatment with these fibrate drugs is caused by the intrinsic properties of the human versus mouse PPARα.

MATERIALS AND METHODS

Animals and Treatments. Mice were maintained under a standard 12-h light/12-h dark cycle with water and chow provided ad libitum. Handling was in accordance with animal study protocols approved by the National Cancer Institute.
Institute Animal Care and Use Committee. Pelleted mouse chow containing 0.1% (w/w) Wy-14,643 or 0.2% (w/w) fenofibrate was prepared by Bioserv (Frenchtown, NJ) and provided to mice ad libitum for 2 or 8 weeks. For the BrdUrd incorporation study, mice were fed Wy-14,643 for 8 weeks, and 1 week before they were killed, mice were implanted s.c. with an Alzet osmotic pump (DURECT Corporation, Cupertino, CA) releasing BrdUrd (16 mg/ml; flow rate, 1 μl/h) as described previously (12). Mice were administered doxycycline (dox; 0–200 μg/ml) in drinking water containing 2% sucrose to regulate expression of human PPARα in the liver. For serum analysis, mice were deprived of food for ~12 h, blood was collected, and then mice returned to the appropriate diet for an additional 3 days before they were killed. Total triglycerides were measured in serum using a commercial kit (Sigma, St. Louis, MO). Body and liver weights were measured after the mice were killed. Tissues not used for histology were snap frozen in liquid nitrogen and stored at −80°C until further analysis. Wy-14,643 was purchased from ChemSyn Science Laboratories (Lenexa, KS); other compounds were purchased from Sigma.

**Generation of Transgenic Mice.** Human PPARα cDNA (14) was cloned into the pTRE2 vector (Clontech Laboratories, Palo Alto, CA), which also contained two direct repeats of insulator sequence (Ref. 21; Fig. 1A). The sequence and orientation were verified using an ABI Prism Big Dye Terminator Kit (Applied Biosystems, Foster City, CA). The TRE-hPPARα transgene was excised from the vector by restriction enzyme digestion and purified before microinjection into FVB/N mouse eggs. Transgene-positive mice were screened by Southern blot analysis and mated to CEBP/β-TA mice expressing the tetracycline-controlled transactivator (tTA) transgene under the control of the liver-enriched activator protein (LAP or CEBP/α)-humanized mice (expressing human PPARα) (Fig. 1A). Mice expressing both transgenes were subsequently bred into mouse PPARα-null transgenic (PPARα−/−; mouse PPARα null transgenic [PPARα-humanized] mice. PCR screening was used to identify tTA (tTA forward, 5’-CTCGCCCAAGAAGCTAGGTGT-3’; tTA reverse, 5’-CCATCGGATGCTTCT-3’, recognizing at 200 bp) and mouse PPARα (mF1, 5’-GAGAGTGCTGCAGAGGGGATGGT-3’; and mR1, 5’-CCATTGCTGACAGGTAGTTCTT-3’; and mNEOR1, 5’-GCAATTCATCTTGTTCAATGGC-3’, recognizing wild-type allele at ~400 bp and the knockout allele at ~650 bp).

**Immunohistochemistry.** BrdUrd immunostaining was performed on liver (and small intestine to verify uniform BrdUrd flow) using an ABC Mouse Vectastain Elite Kit (Vector Laboratories, Burlingame, CA) with a mouse anti-BrdUrd (DakoCytomation, Carpinteria, CA) and an antimouse IgG conjugated to biotin. Immunostaining for catalase was performed on paraffin-embedded liver sections using an ABC Rabbit Elite Vectastain Kit (Vector Laboratories) with a bovine liver catalase antibody (Cortex Biochem, San Leandro, CA) and an antibovine IgG conjugated to biotin. Immunodetection was carried using diaminobenzidine, and sections were counterstained with hematoxylin. The BrdUrd labeling index was determined by counting at least 1500 nuclei/slide (at random high power fields; magnification, 300×) and calculated as 100% × (number of stained hepatocyte nuclei/total number of stained + unstained hepatocyte nuclei).

**Northern and Immunoblot Analysis.** Total RNA was extracted from liver using TRIZol reagent (Invitrogen, Carlsbad, CA). Northern blot analysis was carried out as described previously (23) and hybridized using random primers 5’-labeled cDNA probes (11, 23, 24). Immunoblots of human PPARα were carried out on nuclear extracts prepared using an NE-PER nuclear extraction kit (Pierce, Rockford, IL) and separated on SDS-PAGE using rabbit anti-PPARα (Geneka Biotechnology Inc., Montreal, Canada), antirabbit IgG horseradish peroxidase secondary antibodies (Sigma), and an enhanced chemiluminescence detection kit (Pierce). The polyclonal anti-PPARα antibody recognizes human and mouse PPARα protein. Goat antiactin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as a loading control.

**Statistical Analysis.** All of the values are expressed as the mean ± SD or mean ± SE. All of the data were analyzed by paired or unpaired Student’s t-test for significant differences between the mean values of each group.

**RESULTS**

To explore the species differences in peroxisome proliferator response, PPARα-humanized mice (expressing human PPARα but not mouse PPARα in the liver) were created using a tetracycline responsive regulatory system (25, 26). First, transgenic mice were generated with the human PPARα cDNA fused to the tetracycline response element (TRE-hPPARα; Fig. 1A) and bred with transgenic mice expressing tTA under the control of the liver-specific promoter of CEBP/β (22). The CEBP/β-TA; TRE-hPPARα double transgenic mice were subsequently bred into a mouse PPARα-null background. In the absence of dox, a tetracycline derivative, tTA binds to the TRE and directs transcription of human PPARα specifically in the liver, resulting in production of human PPARα protein. The resulting CEBP/β-TA; TRE-hPPARα mouse PPARα null transgenic mice (now designated as PPARα-humanized mice), which lack mouse PPARα, expressed human PPARα in liver but not in other tissues tested (Fig. 1B). As expected, dox repressed the expression of human PPARα in these mice, as revealed by the absence of human PPARα protein expression in dox-treated PPARα-humanized mice (Fig. 1C). Thus, by using the tetracycline regulatory system, high-level liver expression of human PPARα protein was achieved that was comparable with murine PPARα expression in wild-type mice (Fig. 1B, C; Ref. 22).

The induction of fatty acid oxidation, combined with up-regulation of fatty acid transport, results in a shift in liver fatty acid metabolism with decreased triglyceride synthesis and increased catabolism (1). Studies using PPARα-null mice indicate that the induction of the lipid catabolism genes and decreased serum triglycerides by PPARα agonists are receptor mediated (11, 27). To test the effect of PPARα agonists on liver lipid metabolism in the PPARα-humanized mice, they were fed with the prototypical peroxisome proliferator Wy-14,463 or the clinically used lipid-lowering drug fenofibrate. Wy-14,463 and fenofibrate resulted in decreased serum triglycerides (Fig. 2A), whereas no significant difference was observed in the basal serum triglyceride levels between wild-type and PPARα-humanized mice. Following 2 weeks of Wy-14,463 or fenofibrate feeding, a robust induction of the expression of genes encoding enzymes involved in peroxisomal, mitochondrial, and microsomal fatty acid oxidation was observed.
catabolism (Fig. 2B), as well as those involved in fatty acid synthesis and transport (Fig. 2C), was found in PPARα-humanized mice. Gene responses following Wy-14,643 and fenofibrate feeding were indistinguishable for the genes analyzed (Fig. 3). Administration of dox (200 μg/ml) to the PPARα-humanized mice (thereby repressing human PPARα expression) abolished the effects of Wy-14,643 or fenofibrate on gene expression and triglyceride lowering (data not shown). In vitro transactivation studies have shown similar efficacies of Wy-14,643 and fenofibrate for activating human and rat PPARα, therefore indicating that the extent of drug-induced gene induction is not related to differential maximal activation of human versus mouse PPARα (28). These changes in the expression of genes encoding proteins involved in lipid catabolism are consistent with the triglyceride-lowering effect of Wy-14,643 and fenofibrate in PPARα-humanized and wild-type mice. Hepatomegaly (Fig. 4A) and increases in hepatocyte size (Fig. 4B) were additionally observed in the PPARα-humanized mice fed Wy-14,643 for 2 weeks. Interestingly, the extent of cell size and hepatomegaly was markedly less in PPARα-humanized mice when compared with wild-type mice, especially after 8 weeks of Wy-14,643 feeding (Fig. 4A). Histologically, the livers of wild-type mice treated with Wy-14,643 were composed of greatly enlarged hepatocytes with prominent eosinophilic cytoplasm, which contained small granular structures consistent with the appearance of peroxisomes (Fig. 4B). In sharp contrast, similar cytoplasmic structures could not be definitively seen in H&E-stained sections of either Wy-14,643-treated PPARα-humanized mice or untreated wild-type or PPARα-humanized mice. To support this observation, immunohistochemical staining for the H2O2-degrading enzyme catalase contained within the peroxisome was carried out (Fig. 5). In the wild-type mice treated with Wy-14,643, increased brown granular structures corresponding to catalase-enriched peroxisomes were observed compared with the untreated controls. In comparison, minimal catalase staining was seen in the untreated wild-type mice and untreated and Wy-14,643-treated PPARα-humanized mice; smaller and fewer brown granular structures were observed (Fig. 5). The histologic analysis and

Fig. 2. Peroxisome proliferator response in 2-week-treated peroxisome proliferator-activated receptor α (PPARα)-humanized mice. A, serum total triglycerides. Con, control; WY, Wy-14,643; FF, fenofibrate; hPPARα, human PPARα; and mPPARα, mouse PPARα. Values are mean ± SD (n = 6–9), *P < 0.05 compared with control. B, Northern analysis of fatty acid oxidation genes in liver total RNA using probes as indicated. Microsomal (CYP4A, cytochrome P450 4A family), peroxisomal (ACOX, acyl-CoA oxidase; THIOL, thiolase; BIEN, bifunctional enzyme; and ω-PBE, ω-3-hydroxyacyl-CoA dehydrogenase), and mitochondrial fatty acid oxidation genes (MCAD, medium chain acyl-CoA dehydrogenase; LCAD, long chain acyl-CoA dehydrogenase; VLCAD, very long chain acyl-CoA dehydrogenase; and LCPT, liver carnitine palmitoyltransferase). C, Northern analysis of fatty acid synthesis/transport genes in total liver RNA. ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; ME, malic enzyme; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; FAT, fatty acid translocase; and L-FABP, liver fatty acid binding protein.

Fig. 3. Comparative Wy-14,643 and fenofibrate feeding response in peroxisome proliferator-activated receptor α (PPARα) humanized mice. Northern analysis of liver total RNA. ACOX, peroxisomal acyl-CoA oxidase; VLCAD, very long chain acyl-CoA dehydrogenase; CYP4A, cytochrome P450 4A family; and L-FABP, liver fatty acid binding protein.
catalase immunostaining of the liver sections indicate increased peroxisomes in the wild-type mice treated with Wy-14,643 but not in the Wy-14,643-treated PPARα-humanized mice.

To further examine the mechanism of species difference, we assessed the response of peroxisome proliferator-induced replicative DNA synthesis by measuring BrdUrd incorporation into hepatocyte nuclei after 8 weeks of Wy-14,643 feeding (Fig. 6). Administration of Wy-14,643 for 5 weeks or 11 months reportedly increased replicative DNA synthesis among wild-type but not PPARα null mice (12). The results of immunohistochemistry analysis of BrdUrd-stained hepatocyte nuclei revealed a high degree of incorporation of BrdUrd in Wy-14,643-treated wild-type mouse livers (Fig. 6A) with a labeling index average of 57.9% compared with 1.6% in untreated wild-type controls (Fig. 6B). In PPARα-humanized mice, however, Wy-14,643 treatment did not increase the incorporation of BrdUrd with average labeling indices of 2.8% and 1.6% in Wy-14,643- and control-treated mice, respectively (Fig. 6, A and B). Uniform incorporation of BrdUrd occurs in the small intestine of all mice (Wy-14,643 treated and untreated), thus demonstrating that the lack of increased BrdUrd incorporation into the hepatocyte nuclei of the Wy-14,643-treated PPARα-humanized mice was not because of insufficient BrdUrd labeling in this mouse line (Fig. 6C).

Cyclins and CDKs regulate the transit of cells through the cell cycle. Overexpression of these proteins, as well as PCNA and c-myc, can lead to unregulated cell cycle progression and uncontrolled cell proliferation. These proteins were found previously to be markedly up-regulated in wild-type mice fed Wy-14,643 (29). Consistent with this finding, Wy-14,643 treatment was shown to cause a marked induction in the expression of various genes involved in cell cycle control (PCNA, c-myc, CDK1, CDK4, and cyclins A2, D1, and E) in the livers of wild-type mice (Fig. 6D). However, the expression of these genes was unaffected by Wy-14,643 treatment in PPARα-humanized mice. Conversely, genes encoding peroxisomal, mitochondrial, and microsomal fatty acid oxidation enzymes were still markedly induced in PPARα-humanized mice following 8 weeks of Wy-14,643 feeding (Fig. 6D). Thus, whereas human PPARα regulates induction of fatty acid catabolism and lipid lowering, the species origin of PPARα is important for stimulating the adverse cell proliferative response that contributes to liver carcinogenesis. These results also demonstrate that the effects of PPARα agonists on lipid metabolism are distinct from the effects on hepatomegaly and liver carcinogenesis, thereby suggesting a mechanism by which humans can be resistant to the development of liver cancer but yet still exhibit decreased triglycerides.
DISCUSSION

Oxidative stress and production of reactive oxygen species caused by sustained overproduction of H$_2$O$_2$ and the resulting DNA damage contribute to liver tumor development in rodents (5). However, development of tumors also requires cell proliferation in concert with DNA damage to produce gene mutations. The finding that fibrates do not elevate cell proliferation in PPAR$_\alpha$/H9251-humanized mice (as measured by BrdUrd incorporation and cell cycle gene expression) suggests that these mice would be resistant to liver carcinogenesis induced by long-term peroxisome proliferator treatment because cell proliferation is required for the process of cell transformation. Thus, it would be highly unlikely that the PPAR$_\alpha$-humanized mice would be susceptible to peroxisome proliferator-induced hepatocarcinogenesis.

The differences between the wild-type mice and PPAR$_\alpha$-humanized mice could be caused by differences in ligand affinity between the mouse and human receptors. In vitro transactivation assays previously have shown Wy-14,643 to have higher affinity for mouse or rat PPAR$_\alpha$ than human PPAR$_\alpha$, and thus this could be a factor in the failure to elicit significant alterations in hepatocyte proliferation and the accompanying cell cycle control genes (14, 16). However, Wy-14,643 was capable of inducing several known PPAR$_\alpha$ target genes in the PPAR$_\alpha$-humanized mice, thus indicating that ligand affinity differences between mouse and human PPAR$_\alpha$ may not be important under the conditions used in these experiments. Another possibility that has been considered to account for the differences in response between rats and mice is hepatic levels of PPAR$_\alpha$; mice have much higher levels of expression of the receptor in the liver than do humans (16). However, levels of PPAR$_\alpha$ expression also do not appear to be a factor in the differential response observed in this study because expression of human PPAR$_\alpha$ protein in PPAR$_\alpha$-humanized mice was similar to wild-type mouse PPAR$_\alpha$ levels. These results suggest that the mouse PPAR$_\alpha$ preferentially activates genes required for cell...
proliferation as compared with the human PPARα, a possibility that remains to be investigated. This could be because of differences in cis-acting DR-1 elements between critical target genes required for cell proliferation or differential coactivator recruitment; however, these questions require additional investigation.

Elucidation of the mechanism by which peroxisome proliferators induce carcinogenesis is a prerequisite to assess the toxicologic and health risk to humans in the pharmaceutical use of fibrate lipid-lowering drugs and other drug candidates and chemicals that exhibit peroxisome proliferation in rodent models. Fibrate drugs have been used for >50 years for the treatment of humans with hyperlipidemia, which is a major risk factor for coronary heart disease, without an epidemiologic statistically significant increase in cancer (30–32). However, concerns remain about the risk of cancer in humans exposed to long-term treatments with fibrate drugs and other potentially high-affinity PPARα ligands that are under development to manage hyperlipidemia (15, 33). This holds particular importance because preclinical models used in the pharmaceutical industry are typically rodents.

Although there is no absolutely reliable system other than direct exposure in humans to assess the toxicologic significance of peroxisome proliferators, the development of this PPARα-humanized mouse model provides mechanistic insight into the species differences regarding liver carcinogenesis. As therapies for human diseases become more sophisticated and specifically targeted, it becomes increasingly important to recognize the potential limitations of extrapolating data from mice to humans, and thus by using “humanized” mouse models, the level of uncertainty in extrapolating rodent data to human risk assessment can be reduced. The PPARα-humanized mouse model described in this study should serve as an invaluable tool for predicting cancer risk in humans exposed to drugs that act through PPARα.

This study adds evidence to the idea that the carcinogenic effects of peroxisome proliferators are limited to rodents because of intrinsic differences in the PPARα receptor. Long-term feeding studies with peroxisome proliferators, even high-affinity PPARα ligands, to this mouse model should confirm such conclusions. In summary, the PPARα-humanized mice provide an in vivo platform to facilitate the preclinical evaluation of hepatocarcinogenic risk from the use of fibrates.

ACKNOWLEDGMENTS

We thank Katherine Marsh from the National Cancer Institute and Gary Felsenfeld from the National Institute of Diabetes & Digestive and Kidney Diseases for the plasmid containing pTRE2 with insulator sequences.

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Cancer Res 2004;64:3849-3854.

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