Differential susceptibility of mice humanized for peroxisome proliferator-activated receptor α to Wy-14,643-induced liver tumorigenesis

Keichirou Morimura, Connie Cheung, Jerrold M. Ward¹, Janardan K. Reddy² and Frank J. Gonzalez*²

Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, ¹Comparative Medicine Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA and ²Department of Pathology, Northwestern University School of Medicine, Chicago, IL 60611, USA

*To whom correspondence should be addressed: Laboratory of Metabolism, National Cancer Institute, Building 37, Room 3106, Bethesda, MD 20892, USA. Tel: +1 301 496 9067; Fax: +1 301 496 8419; Email: fjgonz@helix.nih.gov

Peroxisome proliferators, such as lipid-lowering fibrate drugs, are agonists for the peroxisome proliferator-activated receptor α (PPARα). Sustained activation of PPARα leads to the development of liver tumors in rodents. Paradoxically, humans appear to be resistant to the induction of peroxisome proliferation and development of liver tumors by peroxisome proliferators. To examine the species differences in response to peroxisome proliferators, a PPARα humanized mouse (hPPARα) was generated, in which the human PPARα was expressed in liver under control of the Tet-Off system. To evaluate the susceptibility of hPPARα mice to peroxisome proliferator-induced hepatocarcinogenesis, a long-term feeding study of Wy-14,643 was carried out. hPPARα and wild-type (mPPARα) mice were fed either a control diet or one containing 0.1% Wy-14,643 for 44 and 38 weeks, respectively. Gene expression analysis for peroxisomal and mitochondrial fatty acid metabolizing enzymes revealed that both hPPARα and mPPARα were functional. However, the incidence of liver tumors including hepatocellular carcinoma was 71% in Wy-14,643-treated mPPARα mice, and 5% in Wy-14,643-treated hPPARα mice. Upregulation of cell cycle regulated genes such as cd1 and Cdns was observed in non-tumorous liver tissue of Wy-14,643-treated mPPARα mice, whereas p53 gene expression was increased only in the livers of Wy-14,643-treated hPPARα mice. These findings suggest that structural differences between human and mouse PPARα are responsible for the differential susceptibility to the peroxisome proliferator-induced hepatocarcinogenesis. This mouse model will be useful for human cancer risk assessment of PPARα ligands.

Introduction

A variety of chemicals such as hypolipidemic drugs, phthalate ester plasticizers and industrial solvents have been shown to increase the size and number of peroxisomes in rats and mice (1). Because of their unique property of inducing peroxisome proliferation, they are grouped as peroxisome proliferators (2). The hepatic changes caused by treatment with peroxisome proliferators include liver hyperplasia and hypertrophy, proliferation of peroxisomes, and increases in the oxidation of fatty acids through induction of genes encoding mitochondrial, peroxisomal and microsomal enzymes involved in fatty acid oxidation (3,4). Long-term treatment of mice and rats with peroxisome proliferators eventually results in the formation of hepatocellular adenomas and/or carcinomas; these chemicals are regarded as non-genotoxic carcinogens since they do not directly cause genetic damage (5–9).

Peroxisome proliferators exert their effects by activating peroxisome proliferator-activated receptor α (PPARα), a member of the superfamily of ligand-activated, nuclear receptors. In response to ligand activation of PPARα, there is an increase in expression of target genes encoding enzymes and proteins involved in fatty acid transport and catabolism (3). In rodent model systems, PPARα has also been implicated in regulating a number of other biological processes including cell proliferation, apoptosis, inflammation and oxidative stress. Targeted disruption of the mouse Ppara gene confirmed that this receptor is responsible for the peroxisome proliferator-induced pleiotropic responses in mice including the process of hepatocarcinogenesis (5,10).

Notable species differences in response to peroxisome proliferators have been demonstrated (11,12). In spite of their carcinogenic activity in mice and rats, primate and humans appear to be resistant to the induction of peroxisome proliferation and the development of hepatocarcinomas after chronic exposure to the peroxisome proliferator fibrate drugs (13–15). However, humans treated with gemfibrozil, a hypolipidemic drug, for many years have shown no evidence for increased peroxisomes or other pathological alterations found in rodents (14). In addition, epidemiological studies on hypolipidemic drugs showed no increase in cancer incidence (16). Although human PPARα was shown to be functional in transactivation assays (17–19), the mechanism underlying the species difference in response to peroxisome proliferators is still unknown.

Recently, a novel transgenic mouse line humanized for PPARα (hPPARα mice) was generated in order to elucidate the mechanism of species difference in response to peroxisome proliferators (20). These mice express human PPARα specifically in the liver under the control of the doxycycline (Tet-Off) system in a PPARα null background. When treated for up to 8 weeks with the potent peroxisome proliferator, 4-chloro-6-(2,3-xyldino)-pyrimidylnylthio]acetic acid (Wy-14,643), hPPARα mice exhibit decreased serum triglycerides and increased expression of genes encoding...
peroxisomal, mitochondrial and microsomal fatty acid oxidation enzymes, albeit to a lesser extent than wild-type mice. However, unlike wild-type mice, the hPPARα mice did not display increases in ligand-induced hepatomegaly, cell proliferation and expression of cell cycle control genes. These data suggest that the hPPARα mouse may be resistant to peroxisome proliferator-induced hepatocarcinogenesis.

In the present study, to determine the susceptibility of the hPPARα mice to peroxisome proliferators-induced hepatocarcinogenesis, a long-term feeding study of Wy-14,643 was carried out. Expression of some direct PPARα target genes involved in the regulation of the cell cycle was also analyzed.

**Materials and methods**

**Animals and treatments**

A mouse line humanized for the hPPARα was generated as previously described (2). Briefly, a tetracycline response element (TRE) driving human PPARα cDNA transgene (TRE-hPPARA) was microinjected into fertilized FVB/N mouse eggs, and transgene-positive mice were mated to mice expressing the tetracycline-controlled transactivator (tTA) transgene, under the control of the liver-specific Cebpβ promoter (tTALap5Bd). (21). Mice expressing both transgenes were bred into a mouse PPARα-null background (129/Sv/Jae-PPARα−/−) to generate CEBPβ−/−; tTA; TRE-hPPARα; PPARα−/− transgenic mice (129/Sv/Jae-Cg-PPARα−/−; Tg[TRE-hPPARα]1Gonz Tg(tTALap5Bd). This mouse line expresses human PPARα cDNA in the liver hepatocytes on a mouse PPARα-null background in the absence of doxycycline, a tetracycline derivative (Tet-OFF system). The genotypes for all animals used in this study were verified using PCR detection of tTA (tTA forward, 5'-CTCGCCAGAAGCTAGGTGT-3'; tTA reverse, 5'-CCATCGGCAGTACCTAGGT-3', recognizing at 200 bp), mouse PPARα (m01F1, 5'-GAGAAGTGGCAAGGGAGTTGTG-3'; m01R1, 5'-CCCATTTCGGTGACATCCTTCT-3'; and m01R01, 5'-GCCATCTCATCCTGTTCAATGGC-3', recognizing wild-type allele at ~400 bp and the null allele at ~650 bp), and human PPARα (hPPAR2 GENO F3, 5'-CGATTITCCACAACTGCTTTCGTCC-3'; hPPAR2 GENO R3, 5'-AAATTTCCGGAGCGCTGGAGTGG-3', recognizing at 430 bp).

A total of 30, 6-week-old male hPPARα mice were divided into two groups and 10 and 20 mice were given a pelleted diet with or without 0.1% (w/w) Wy-14,643 (Geneka Biotechnology, Montebello, CA, USA). Northern blot analysis was carried out as described previously (23) and hybridized with random primer [32P]-labeled cDNA probes (23,24) and exposed to a PhosphorImager screen cassette followed by visualization using a Storm 860 PhosphorImager system (Amersham Biosciences, NJ, USA). Signals were quantified using ImageQuant TL software (Amerham Biosciences). The expression levels of target genes were normalized with those of reference gene (acidic ribosomal phosphoprotein; 36B4). Five mice each per group were subjected to the analysis.

**Statistical analysis**

Survival curves were created using the Kaplan–Meier method, and the statistical significance of differences was calculated by the log-rank test. Variations in liver/body weight ratio, incidences of tumors, gene expression analysis between the different treatments or animal strains were evaluated with one-way factorial ANOVA and multiple comparison tests. All the calculations for statistical analysis were performed using the Statview SE+ Graphics, version 5.0 (Abacus Concepts, Berkeley, CA, USA).

**Results**

**Survival, body and liver weights**

From the onset of the experiment, Wy-14,643-treated mPPARα mice exhibited suppressed growth compared with all other groups and the percentage of animals of this group that survived decreased to 50% at the termination of the study period (Figures 1 and 2). Of the 10 mice in the Wy-14,643-treated mPPARα group, 3 died of toxicity and were not evaluated further whereas 2 mice were killed due to morbidity before the termination of the experiment. However, the Wy-14,643-treated hPPARα mice showed a similar rate of growth as those of the non-treated control mice of both strains, and only one animal in this group was killed due to morbidity before termination of the experiment (Figures 1 and 2).

The final body weight of Wy-14,643-treated mPPARα mice was markedly decreased and both absolute and relative liver weights increased as compared with those of all other groups (Table I). Wy-14,643-treated hPPARα mice showed an increased absolute and relative liver weights compared with the corresponding control diet group; however, the extent of increase was considerably less that found in the Wy-14,643-treated mPPARα mice (Table I).

![Fig. 1. Time-course changes in mean body weights. Mice were weighed every 2 weeks throughout the course of the study. The mPPARα mice + Wy-14,643 group showed a lower rate of growth from the onset of the experiment.](image-url)
Hepatic lesions
Most of the mPPARα mice fed the Wy-14,643 diet had multiple, grossly visible nodules that were randomly distributed among the liver lobes (Figure 3A). The hepatocellular adenomas were well-circumscribed lesions compressing adjacent parenchyma without normal lobular architecture, and were composed of well-differentiated hepatocytes (Figure 3E). The hepatocellular carcinomas were characterized by a trabecular or pseudoglandular histologic pattern (Figure 3F).

Table II shows incidence of the hepatocellular adenoma/carcinoma and pre-neoplastic altered cell foci in the liver of the various groups. The incidence of hepatocellular adenomas in the Wy-14,643-treated mPPARα mice was 71% and two animals had hepatocellular carcinomas. Most animals in this group with tumors had single or multiple altered cell foci (Figure 3B and C). In contrast, only 1 out of 20 hPPARα mice treated with Wy-14,643 harbored a hepatocellular adenoma, and significant differences in the incidence was detected as compared with Wy-14,643-treated mPPARα mice. This tumor was also morphologically similar to spontaneous mouse liver tumor with basophilic and clear hepatocytes, whereas the tumors in mPPARα mice were more diffusely basophilic. Altered cell foci were detected in one hPPARα animal in each of the mice with and without Wy-14,643 treatment. Histologically, toxic lesions induced by Wy-14,643 were seen in mPPARα mice and were much less severe in hPPARα mice (Figure 3B–D).

Table I. Final body and absolute/relative liver weights

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Effective no. of mice</th>
<th>Final body weight (g)</th>
<th>Liver weight (g): Absolute (%)</th>
<th>Relative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPPARα</td>
<td>Control diet</td>
<td>10</td>
<td>39.2 ± 7.9b</td>
<td>1.57 ± 0.4</td>
<td>3.96 ± 0.4</td>
</tr>
<tr>
<td>hPPARα</td>
<td>Wy-14,643</td>
<td>20</td>
<td>40.3 ± 6.1</td>
<td>2.37 ± 0.5*</td>
<td>5.87 ± 0.7*</td>
</tr>
<tr>
<td>mPPARα</td>
<td>Control diet</td>
<td>9</td>
<td>33.9 ± 6.4</td>
<td>1.10 ± 0.1</td>
<td>3.29 ± 0.4</td>
</tr>
<tr>
<td>mPPARα</td>
<td>Wy-14,643</td>
<td>7</td>
<td>16.4 ± 2.9**</td>
<td>3.03 ± 1.4**</td>
<td>17.86 ± 5.6***</td>
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*P < 0.05 versus hPPARα + Control; **P < 0.05 versus all other groups; ***P < 0.0001 versus all other groups.

Hepatic lesions
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PPARα expression
At the termination of the carcinogenicity study, both gene and protein expression levels of PPARα were evaluated using the non-tumorous portion of liver tissue (Figure 4). Total RNAs were hybridized with specific probes for human and mouse PPARα genes independently for northern blot analysis. Both hPPARα and mPPARα mice showed expression of the respective human and mouse PPARα mRNA regardless the treatment.

Fig. 2. Time-course changes in survival rates. Mice were examined twice a week and those exhibiting distress were killed. Surviving curves were created using the Kaplan–Meier method. *P < 0.05 versus all other groups (log-rank test).

Table II. Incidences of liver lesions

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Effective no. of mice</th>
<th>Altered foci (%)</th>
<th>Tumor (%)</th>
</tr>
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<tbody>
<tr>
<td>hPPARα</td>
<td>Control diet</td>
<td>10</td>
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<tr>
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<td>7</td>
<td>5 (71)</td>
<td>5 (71)</td>
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*P < 0.01 versus values of hPPARα + Wy-14,643.

Fig. 3. Representative photomicrographs of liver lesions. (A) A grossly visible hepatic nodular mass is seen in the liver of a wild-type mouse after 38 weeks of Wy-14,643 feeding. (B) Photomicrograph of the eosinophilic altered cell foci in a wild-type mouse after 38 weeks of Wy-14,643 feeding. (C) High magnification of (B) shows severe toxic lesions including large eosinophilic hepatocytes with eosinophilic granular cytoplasm, pigment accumulation within Kupffer cells (arrows) and small basophilic cells of proliferating oval cells. (D) A photomicrograph of livers from a humanized PPARα mouse after 44 weeks of Wy-14,643 feeding. Mild fatty change and glycogen deposition are seen, but the toxic lesions are not evident compared with liver from wild-type mice after 38 weeks of Wy-14,643 feeding. (E) and (F) A photomicrograph of a representative hepatocellular adenoma and carcinoma in wild-type mouse after 38 weeks of Wy-14,643 feeding, respectively.

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*P < 0.05 versus hPPARα + Control; **P < 0.05 versus all other groups; ***P < 0.0001 versus all other groups.

aLiver wt/body wt (%).

bMean ± SD.
of Wy-14,643 (Figure 4A). Moreover, PPARα proteins were also expressed similarly in hPPARα and mPPARα mice (Figure 4B).

**Gene expression analysis**

To examine the expression of PPARα target genes and genes involved in cell cycle/apoptosis, northern blot analyses were carried out (Figure 5A), using non-tumorous portions of liver collected at the termination of the animal experiment, and quantification of expression normalized with a reference gene (Figure 5B–D). Expression levels of acyl-CoA oxidase (ACOX), cytochrome P450 A (CYP4A) and medium chain acyl-CoA dehydrogenase (MCAD) mRNAs in both Wy-14,643-treated hPPARα and mPPARα mice were significantly increased, compared with those of corresponding groups fed the control diet (Figure 5B). mRNA encoding malic enzyme (ME) was significantly increased in the Wy-14,643-treated mPPARα group, compared with that of the control diet group, but no difference was detected between Wy-14,643- and control-diet-treated hPPARα mice groups. Figure 5C shows the expression level of genes regulating cell cycles. mRNAs encoding CD1, cyclin-dependent kinases (CDKs) 1 and 4 were highly expressed in the livers of Wy-14,643-treated mPPARα mouse group and were statistically different from those in all other groups. The cMYC mRNA was also significantly overexpressed in the Wy-14,643-treated mPPARα group compared with those of control-diet-treated mPPARα group, but no difference was detected compared with those of other groups. The mRNAs encoding apoptosis associated proteins, p53, p21, BAX and BCL2 were also examined (Figure 5D). Expression of the p53 gene was increased in the Wy-14,643-treated hPPARα group as compared with those of all other groups, although the P-value between Wy-14,643-treated mPPARα was marginal (P = 0.416). The p21 mRNA showed a slight but not statistically significant increase in the Wy-14,643-treated hPPARα. No difference was found in expression of mRNAs encoding BAX and BCL2 among all groups.

**Discussion**

Chronic dietary exposure of mice and rats to Wy-14,643 and other peroxisome proliferators typically results in hepatocellular neoplasia (5,7–9). In the present study, a high incidence of hepatocellular tumors (71%) including hepatocellular carcinomas was detected in mPPARα, fed with 0.1% Wy-14,643 for up to 38 weeks. Owing to the moribund status of mice in this group, Wy-14,643 treatment was prematurely terminated at 38 weeks from the beginning of the experiment. In sharp contrast, hPPARα mice given the same diet for up to 44 weeks, at which the wild-type mice exhibited a 100% incidence of liver tumors under the same treatment (5), demonstrated a very low incidence of hepatocellular tumors (5%). This observation represents conclusive evidence for low-susceptibility of this humanized mouse model to peroxisome proliferator-induced hepatocarcinogenesis.

In the presence of ligand, PPARα heterodimerizes with RXRs and binds to peroxisome proliferator response elements (PPREs) in the promoter region of target genes, resulting in increased transcription and expression of proteins and enzymes necessary for the transport and catabolism of fatty acids. A large number of genes including peroxisomal (CoxS), microsomal (Cyp4a) and mitochondrial (Mcad) fatty acid oxidation genes, as well as fatty acid synthase (Fas) gene are well documented as PPARα target genes (25). In this study, the expression of most of these target genes were elevated in the Wy-14,643-treated hPPARα mouse, revealing that hPPARα was functional in control of fatty acid homeostasis similar to the wild-type mouse and that transcription was continuously stimulated throughout the course of ligand treatment.

**Cyclins and CDKs** regulate the cell cycle and overexpression of these proteins can result in uncontrolled cell proliferation. These proteins were previously found to be markedly upregulated in wild-type mice fed Wy-14,643 (26). Consistent with these findings, a number of cell cycle control genes were also upregulated in the mPPARα mouse group fed Wy-14,643, whereas the hPPARα mouse, under the same treatment regimen, did not show increased expression of these genes. These results indicate that genes associated with cell proliferation are preferentially activated in the livers of wild-type mice as compared with the hPPARα mouse line or that the severe toxicity and/or hepatocyte damage in the wild-type mouse results in induction of cell cycle control genes associated with the hepatocytic regenerative response. However, whether an increase in the expression of these genes is a cause or an effect of carcinogenesis is not elucidated in this study, because the total RNAs used in this analysis were derived from liver tissues of mice chronically exposed to Wy-14,643.

In response to the ligand activation, a number of lipid-metabolizing enzymes are induced in rodents. Increased levels of enzymes associated with the peroxisomal fatty acid β-oxidation system including acyl-CoA oxidase and to a lesser degree, the microsomal CYP4A subfamily of enzymes involved in fatty acids ω-oxidation leads to the generation of hydrogen peroxide (H2O2) (27). Increased H2O2 could potentially react with metals and generate highly reactive hydroxyl radicals, or react with lipid resulting in lipid peroxides ultimately elevating reactive oxygen species (ROS) and the degree of oxidative stress that can cause damage to DNA, protein, lipids and other cellular components that contribute to hepatocarcinogenesis in rodents (28). However, ROS may also act as a second intermediate in intracellular signaling (29) or inhibit the
mitochondria respiratory chain complex leading to apoptosis (30,31). Although suppression of apoptosis has been implicated as a mechanism for hepatocarcinogenicity of peroxisome proliferators, these compounds can induce cell death in the human HepG2 cell line (32). A peroxisome proliferator, BR931, was also demonstrated to induce apoptosis through a p53-dependent pathway in the rat hepatoma FaO cell line (33). Overexpression of the p53 gene in Wy-14,643-treated hPPARα mice may imply that a sustained activation of the programmed cell death system contributes to the resistance to hepatocarcinogenesis, in contrast to the Wy-14,643-treated mPPARα mice group with comparatively lower expression of the p53 gene. However, the mutated form of this protein is retained in the cell, resulting an abnormal cell cycle. Besides, it should be noted that hepatoma cells lines are markedly different from hepatocytes and thus the results obtained with a rat hepatoma FaO cell line may not reflect the activity of peroxisome proliferators in vivo. Additional studies are necessary to

Fig. 5. Northern blot analysis of PPARα target genes and cell cycle/apoptosis regulating genes using probes as indicated (A). Total RNA (10 μg/lane) was isolated from livers of non-cancerous tissue (n = 5 animals) from each animal. The signal for acidic ribosomal phosphoprotein (36B4) was used as a control for loading and RNA integrity. Cont., control diet; Wy-14,643, control diet containing 0.1% Wy-14,643; ACOX, acyl-CoA oxidase; CYP4A, cytochrome P450 4A family; MCAD, medium chain acyl-CoA dehydrogenase; ME, malic enzyme; cMYC, c-myc; CD1, cyclin D1; CDK, cyclin-dependent kinase. Quantification analysis of gene expression (B, C and D). All values were normalized to the signal for 36B4. Results represent mean ± SD. (B) Results for PPARα target genes. *P < 0.05, **P < 0.01 versus corresponding to mice fed with control diet. (C) Results for cell cycle regulating genes. *P < 0.05, **P < 0.01 versus mPPARα fed with diet containing 0.1% Wy-14,643. (D) Results for apoptosis regulating genes. *P < 0.05, **P < 0.01 versus hPPARα fed with diet containing 0.1% Wy-14,643.
establish a role for p53 in the resistance of the hPPARα mice to hepatocarcinogenesis.

It must be considered that hepatic levels of PPARα and/or different ligand affinity of receptor could account for the differential response in cell proliferation between mice and humans as revealed in the altered expression of cell cycle control genes involved in hepatocyte proliferation and carcinogenesis. Indeed, transient retroviral overexpression of the human PPARα in PPARα-null mice resulted in induction of some target genes accompanied by increased cell proliferation, indicating the higher level of human PPARα may contribute liver cell proliferation (19). A different ligand affinity for Wy-14,643 between human and mouse PPARα was also reported (34). However, the level of PPARα expression does not appear to be critical in this model, because the cellular content of human PPARα protein in the hPPARα mice detected in this study was similar to the mouse PPARα protein levels in wild-type mice. Furthermore, similar expression levels of several potent PPARα target genes such as ACOX and CYP4A were found comparing the wild-type and PPARα mice, indicating that the ligand affinity differences between human and mouse PPARα may not be important under the conditions used in these experiments. It also cannot be ruled out that the hPPARα mice are resistant to the hepatotoxic effects of peroxisome proliferators due to the site of expression of the human receptor. The cDNA was placed under control of the tetracycline regulatory system and the liver-specific Cebpβ promoter that is preferentially expressed in hepatocytes. Thus, it cannot be excluded that expression of PPARα in other cells in the liver such as Kupffer or stellate cells may be involved in the hepatoproliferative response. However, PPARα is not expressed at appreciable levels in Kupffer cells (35).

The rodent model is widely accepted as a useful tool for evaluating chemical carcinogenicity and/or toxicity, in accordance with the recommendation of the International Conference on Harmonization of Technical Requirements of Pharmaceuticals for Human Use (ICH). However, for assessment of the hazards of peroxisome proliferators, including hypolipidemic drugs, to human health, an explanation for the interspecies differences to those chemicals yields some important insights. Although the epidemiological studies on long-term treatment with hypolipidemic drugs showed inconclusive evidence of carcinogenic effects in humans (16), elucidation of the mechanisms by which peroxisome proliferators induce carcinogenesis is a prerequisite to accurately assess the human risk. In this regard, the hPPARα mouse model will provide a valuable model for determining the mechanism of the species differences in liver carcinogenesis and the mechanism of action of non-genotoxic hepatocarcinogens.

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