PPAR α -Dependent Activation of Cell Cycle Control and DNA Repair Genes in Hepatic Nonparenchymal Cells

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Peroxisome proliferator-activated receptor- α (PPAR α) mediates the diverse biological effects of peroxisome proliferator (PP) chemicals, including fatty acid catabolism, hepatomegaly, hepatocyte proliferation, and hepatocarcinogenesis in rodents. However, transgenic mice expressing a constitutively active PPAR α in hepatocytes (VP16PPAR α) do not develop hepatocellular carcinomas in spite of hepatocyte proliferation and hepatomegaly; this suggests that activation of genes in nonparenchymal cells may have a critical role in PP-induced carcinogenesis. VP16PPAR α mice exhibited massive peroxisome proliferation and hepatomegaly as well as increased mortality upon Wy-14,643 treatment. Several genes involved in cell cycle or DNA damage repair, such as Chek1, Prkdc, Mcm, and Rad51, were significantly induced to a similar extent between wild-type and VP16PPAR α mice after Wy-14,643 administration. This induction was completely abolished in *Ppar α* -null mice, suggesting a PPAR α -dependent pathway. These data revealed a DNA damage response signaling network as an early event upon PP treatment and provide novel putative mechanisms for PP-induced hepatocellular carcinoma.

Key Words: PPAR α ; peroxisome proliferators; hepatomegaly; DNA damage repair; carcinogenesis.

INTRODUCTION

Peroxisome proliferators (PPs) represent a structurally diverse group of agents that can cause peroxisome proliferation in rodents, including the fibrate hypolipidemic drugs, phthalate ester plasticizers, and herbicides (Kersten and Wahli, 2000). Exposure of rodents to PPs leads to hepatomegaly, peroxisome proliferation, and an increase of fatty acid catabolism as a result of enhanced expression of genes involved in lipid transport and fatty acid β -oxidation (Lazarow and De Duve, 1976; Reddy and Krishnakantha, 1975). Chronic administration of PPs results in 100% incidence of liver tumors in rodents (Reddy *et al.*, 1980). However, the molecular mechanism is not completely understood.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily, and three isoforms of PPARs (α , β/δ , and γ) have been identified (Dreyer *et al.*, 1992; Issemann and Green, 1990). PPARs act as transcription factors via a classic ligand-dependent nuclear hormone receptor mechanism. Upon binding to their ligands, PPARs undergo conformational changes that allow corepressor release and coactivator recruitment, heterodimerization with retinoid X receptor, and selective binding to specific sequences termed PP response elements in the promoter of target genes. PPAR α , first cloned, identified, and named based on its ability to be activated by PPs (Issemann and Green, 1990), serves a central role in maintaining lipid homeostasis by activating genes encoding peroxisomal and mitochondrial enzymes involved in fatty acid metabolism (Berger and Moller, 2002).

Chronic treatment with PPAR α agonist induces liver tumors via a PPAR α -dependent mechanism as revealed by the resistance of *Ppar α* -null mice to liver cancer (Hays *et al.*, 2005; Peters *et al.*, 1997). However, the precise molecular mechanism leading to PPAR α -induced hepatocarcinogenesis has not been fully uncovered. Recently, transgenic mice expressing a constitutively active hepatocyte-specific PPAR α (VP16PPAR α mice) were established using the potent viral transcriptional activator VP16 fused to mouse PPAR α complementary DNA (cDNA) under the control of liver-enriched activator protein (LAP) promoter (Yang *et al.*, 2007). In the absence of PPAR α ligands, these mice exhibit multiple hepatic responses similar to wild-type mice treated with PPs, including a decrease in serum triglycerides and free fatty acids, a massive increase in peroxisome number, and marked induction of PPAR α target genes encoding fatty acid β -oxidation enzymes. However, the transgenic mice at 1 year old demonstrated no gross or microscopic evidence of either preneoplastic hepatic lesions or hepatocellular neoplasia that are evident in wild-type mice treated with PP. These results indicate that PPAR α activation only in hepatocytes is not

sufficient to induce tumorigenesis in the liver. In the current study, the effects of Wy-14,643 on hepatocyte proliferation and gene expression patterns were compared between VP16PPAR α and wild-type mice.

METHODS AND MATERIALS

Animals. Mice with hepatocyte-restricted PPAR α activation in the absence of ligand (VP16PPAR α) were generated as described previously (Yang *et al.*, 2007). Briefly, the potent viral transcriptional activator VP16 was fused to mouse PPAR α cDNA to constitutively activate PPAR α -responsive genes in the absence of ligand. Then, tetracycline response element was fused to VP16PPAR α to generate TREVP16PPAR α transgenic mice. Subsequently, TREVP16PPAR α mice were bred with mice expressing the tetracycline-controlled transactivator under the control of LAP to obtain the double transgenic mice (referred to herein as VP16PPAR α mice). The transgenic mice were genotyped by PCR of tail DNA as described previously (Gossen and Bujard, 1992; Yang *et al.*, 2006). PPAR α -null male mice (SV129 background; Lee *et al.*, 1995) and corresponding wild-type counterparts (6 weeks old at the beginning of treatment) were used in these experiments.

Animal treatments. Mice were housed under a standard 12-h light-dark cycle with water and chow provided *ad libitum*. For Wy-14,643 treatment, mice were administered with Wy-14,643 (0.1% [wt/wt]; Bio-Serv, Frenchtown, NJ) in the diet for indicated times. Mouse handling and all experimentation were in accordance with animal study protocols approved by the Animal Care and Use Committee at National Cancer Institute.

Histological analysis. Mice were killed by overexposure to carbon dioxide and livers excised, fixed in 10% neutral-buffered formalin (Fisher Scientific), embedded in paraffin, and 4- μ m sections were prepared. Sections were stained with hematoxylin and eosin and were evaluated by light microscopy.

RNA analysis. Total RNA was isolated from livers with TRIzol reagents (Invitrogen, Carlsbad, CA). Quantitative real-time PCR (qPCR) was performed using cDNA generated from 1 μ g of total RNA with SuperScript II Reverse Transcriptase Kit (Invitrogen). qPCRs were carried out by use of SYBR Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Values were quantified by comparative C_t methods and normalized to β -actin. The sequences for the primers used to quantify messenger RNA (mRNA) are listed in Supplementary table 1.

cDNA microarray analysis. Dye-coupled cDNAs were purified with a MiniElute PCR Purification Kit (QIAGEN) and hybridized to an Agilent 44 K mouse 60-mer oligo microarray (Agilent Technologies, Santa Clara, CA). The procedures were repeated for replicate experiments with independent hybridization and processing. The data were processed and analyzed by Genespring GX software (Agilent Technologies).

Data analysis. All data were presented as mean $\pm$ SD. Differences between groups were assessed by one-way ANOVA. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Wy-14,643 Exacerbated Hepatomegaly in VP16PPAR α Mice

A fourfold increase in PPAR α mRNA expression was found in the livers of VP16PPAR α mice (Fig. 1A). Western blot analysis revealed one high-mobility band from wild-type mice and two bands, corresponding to the endogenous and the low-

mobility transgenic VP16PPAR α fusion protein (Fig. 1B). Antibodies against VP16 could detect one band only from the transgenic group. These results are consistent with previous findings that both endogenous PPAR α and transgenic VP16PPAR α are detectable by northern blot analysis in the transgenic mice (Yang *et al.*, 2007). Transgenic VP16PPAR α mice exhibit hepatomegaly in the absence of PP exposure. To explore the responses of transgenic mice to PPs, wild-type and VP16PPAR α mice were treated with Wy-14,643 for 5 days. Compared with untreated transgenic mice and Wy-14,643-treated wild-type mice, Wy-14,643 treatment resulted in a more significant weight loss in transgenic VP16PPAR α mice (Fig. 1C). Furthermore, the liver/body weight ratio was markedly increased in these mice (Fig. 1D). Histological analysis of liver sections revealed that transgenic mice treated with Wy-14,643 had dramatic hepatocyte hypertrophy and peroxisome proliferation (Fig. 1E). These data suggest that PPAR α activation of VP16PPAR α mice by Wy-14,643 exacerbates the hepatomegaly. Because hepatocyte-specific PPAR α activation only leads to hepatocyte proliferation and not liver tumors, the susceptibility of VP16PPAR α mice to Wy-14,643-induced hepatocellular carcinoma was investigated. Unexpectedly, 80% of transgenic mice receiving Wy-14,643 died within 1 month; no mortality was observed in wild-type mice receiving Wy-14,643 or the VP16PPAR α group without Wy-14,643 (Fig. 1F). Coincident with the decreased survival rate is the severe hepatomegaly as revealed by liver/body weight ratio (Fig. 1G). These data indicate that PPs may induce severe liver toxicity in the VP16PPAR α mice.

PPAR α Target Gene Induction in Wy-14,643-Treated VP16PPAR α Mice

PPAR α activation regulates the transcription of numerous genes involved in fatty acid transport and oxidation. The VP16PPAR α transgene in hepatocytes can induce the expression of genes encoding enzymes involved in fatty acid catabolism. Upon Wy-14,643 challenge, these genes, including acyl-CoA oxidase (Acox1), cytochrome P450 family 4a14 (Cyp4a14), and Cyp4a10, were additionally induced compared with untreated VP16PPAR α mice, suggesting that VP16PPAR α activity is comparable to Wy-14,643-liganded native PPAR α in hepatocytes (Fig. 2).

Regulation of Genes Involved in Hepatocyte Proliferation and Cell Cycles by Wy-14,643 in VP16PPAR α Mice

Both hepatocyte and nonparenchymal cell (NPC) proliferation are important components for PP-induced hepatocellular carcinoma (Hasmall *et al.*, 2001; Yang *et al.*, 2007). Proliferating cellular nuclear antigen mRNA, a marker for cell proliferation, was upregulated in Wy-14,643-treated VP16PPAR α livers compared with untreated transgenic mice or 5-day Wy-14,643-treated wild-type mice, respectively (Fig. 3A), suggesting an enhanced cell proliferation in these

FIG. 1. Wy-14,643 aggravates hepatomegaly in VP16PPAR α transgenic mice. (A) qPCR analysis for PPAR α mRNA expression in the liver of wild-type and VP16PPAR α mice. (B) Western blotting for nuclear VP16 and PPAR α protein in the liver of wild-type and VP16PPAR α mice. (C) Changes of body weight in wild-type and VP16PPAR α mice after Wy-14,643 treatment for 5 days. (D) Liver/body weight ratio in wild-type and VP16PPAR α mice after Wy-14,643 treatment for 5 days. (E) Hematoxylin and eosin staining of liver sections from wild-type and VP16PPAR α mice treated by Wy-14,643 for 5 days. (F) Survival curve of wild-type and VP16PPAR α mice when receiving Wy-14,643 diet for 1 month; $n = 8-10$ per group. (G) Liver/body weight ratio of wild-type and VP16PPAR α mice at the end point of 1-month Wy-14,643 treatment. * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ versus WT-Con group.

mice. Interestingly, a key oncogene cMyc, which is regulated by PPAR α target miRNA let-7C after Wy-14,643 exposure (Shah *et al.*, 2007), was increased only in wild-type or VP16PPAR α mice treated with Wy-14,643 compared with

untreated wild-type or VP16PPAR α mice (Fig. 3B). Because the VP16PPAR α transgene is exclusively expressed in hepatocytes, these results suggest that PPs induce a subset of genes that are critical for tumorigenesis in NPC.

FIG. 2. Induction of PPAR α target genes by Wy-14,643 treatment in wild-type and VP16PPAR α transgenic livers. qPCR analysis for fatty acid oxidation genes in liver from 6- to 8-week-old wild-type mice after Wy-14,643 administration for 5 days. Microsomal (Cyp4a10 and Cyp4a14, cytochrome P450 4A family) and peroxisomal (Acox, acyl-CoA oxidase) fatty acid oxidation gene expression levels were normalized to β -actin and values were mean $\pm$ SD ($n = 4-6$). ** $p < 0.01$ compared with wild-type control.

Gene Expression Profiling in Wild-Type and VP16PPAR α Transgenic Mice Livers following Wy-14,643 Treatment

A previous study revealed that hepatocyte-restricted PPAR α activation is sufficient to induce hepatocyte proliferation but insufficient for hepatocellular carcinoma, thus indicating an involvement of NPC activation in the development of Wy-14,643-induced liver tumors (Yang *et al.*, 2007). To fully address the gene expression profile induced by Wy-14,643 treatment, microarray analysis was performed in liver samples from 5-day Wy-14,643-treated or untreated wild-type and VP16PPAR α mice. As listed in Table 1 (Supplementary table 2), the most significantly upregulated genes after Wy-14,643 treatment or untreated VP16PPAR α mice were classical PPAR α target genes involved in fatty acid or lipid metabolism, including Cyp4a14, fatty acid-binding protein 4 (Fabp4), Cyp4a10, Fabp3, lipoprotein lipase (Lpl), Acot1, carnitine acetyltransferase (Crat), Cd36, elongation of long chain fatty

FIG. 3. Changes of cell proliferation-related or cell cycle-related genes in the liver from wild-type or VP16PPAR α mice exposed to Wy-14,643 for 5 days. qPCR analysis for cell proliferation marker proliferating cellular nuclear antigen and cMyc. * $p < 0.05$ versus wild-type group or VP16PPAR α .

acids family 7 (Elovl7), and cell death-inducing DFFA-like effector c (Cidec), consistent with data in Figure 2.

To gain additional insights into the Wy-14,643-induced events that link endogenous PPAR α activation, cell proliferation, and liver tumor development, microarray data were further analyzed to identify genes that demonstrated exclusive activation following Wy-14,643 administration in livers from wild-type and VP16PPAR α mice. Several genes were identified, and a significant numbers of genes were related to cell cycle or DNA damage repair, such as checkpoint kinase 1 homologue (Chek1), protein kinase, DNA activated, catalytic polypeptide (Prkdc), RAD51, minichromosome maintenance deficient 2 mitotin (Mcm2), Mcm5, Mcm8, and Mcm7. The above gene expression data were confirmed by qPCR analysis (Table 2, Fig. 4A).

To further elucidate whether the induction of the above genes by Wy-14,643 is PPAR α dependent, Wy-14,643 was administered to *Ppar α* -null mice or the wild-type controls for 5 days and then qPCR was performed. As shown in Figure 4B, all the above genes, including Prkdc, Chek1, Mcm2, Mcm5, and Cyclin D1, were robustly induced in wild-type mice after Wy-14,643 treatment, whereas no induction was noted in Wy-14,643-exposed *Ppar α* -null mice. These data demonstrate that induction of the above genes is PPAR α dependent and provides putative target genes that may be critical to fibrate-induced carcinogenesis.

DISCUSSION

PPs including fibrates are nongenotoxic liver carcinogens in rodents. However, the molecular links between PPs and tumorigenesis have not been fully uncovered. In the current study, a DNA damage response signaling network, especially Chek1 and its checkpoint signaling cascades, was found to be critical to PP-induced hepatocyte proliferation and hepatocarcinomas. Although fibrates have been widely used for lowering plasma triglycerides for many years, short-term exposure of rodents to these drugs initiates a pleiotropic response of hepatomegaly, peroxisome proliferation, and increased fatty acid catabolism in liver, whereas long-term administration results in hepatocellular carcinomas. The *Ppar α* -null mouse model revealed that PPAR α is required for the pleiotropic responses (Lee *et al.*, 1995; Peters *et al.*, 1997). However, the mechanism by which PPAR α activation increases the incidence of hepatocellular carcinomas in rodents was not clear. Previously, characterization of the VP16PPAR α mice demonstrated that activation of PPAR α only in hepatocytes is sufficient to decrease triglycerides and induce hepatocellular proliferation but not liver tumors (Yang *et al.*, 2007). These data suggest that activation of PPAR α only in hepatocytes is not sufficient to initiate liver tumorigenesis and indicate that activation of PPAR α in other liver cell types is also critical to PP-induced hepatocarcinomas. The most important difference

TABLE 1
Classical PPAR α Target Genes Differentially Regulated by Wy-14,643 in Wild-Type (WT) and VP16PPAR α Mice

Accession number	WT	WT + Wy (folds)	VP16PPAR α (folds)	VP16PPAR α + Wy (folds)	Gene description
NM_007822	1	99.92995	74.51917	250.0674	Cytochrome P450, family 4, subfamily a, polypeptide 14 (Cyp4a14)
NM_024406	1	54.67093	24.61459	593.5192	Fatty acid-binding protein 4, adipocyte (Fabp4)
NM_010011	1	32.23628303	15.24794	34.59521	Cytochrome P450, family 4, subfamily a, polypeptide 10 (Cyp4a10)
NM_008655	1	27.57955	10.35417	23.64452	Growth arrest and DNA damage-inducible 45 beta (Gadd45b)
NM_023737	1	19.91719	12.05095	20.7529	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase (Ehhadh)
NM_010174	1	18.9171	7.413726	24.81403	Fatty acid-binding protein 3 (Fabp3)
NM_008509	1	14.86413	9.913214	19.08175	Lipoprotein lipase (Lpl)
NM_012006	1	14.83032	8.479407	37.3034	Acyl-CoA thioesterase 1 (Acot1)
NM_007760	1	14.08509	8.101722	12.13041	Carnitine acetyltransferase (Crat)
NM_007643	1	13.45972	5.096111	18.53204	CD36 antigen (Cd36)
NM_029001	1	13.3322	7.702144	21.4951	ELOVL family member 7, elongation of long chain fatty acids (Elovl7)
NM_178373	1	12.90596	10.91521	8.848053	Cell death-inducing DFFA-like effector c (Cidec)
NM_013743	1	12.36	8.5687	16.70559	Pyruvate dehydrogenase kinase, isoenzyme 4 (Pdk4)
NM_015729	1	7.480115	7.383852	13.39764	Acyl-Coenzyme A oxidase 1, palmitoyl (Acox1)

between VP16PPAR α transgenic mice and PP-treated wild-type counterpart is the absence of endogenous PPAR α activation in the transgenic mice. The current study sought to compare the responses of VP16PPAR α mice to Wy-14,643 with that of wild-type mice upon Wy-14,643 treatment. Peroxisome proliferation, hepatocyte proliferation, and hepatomegaly were greatly exacerbated by short-term Wy-14,643 treatment in transgenic mice. Moreover, a 1-month Wy-14,643 administration was sufficient to induce a striking hepatomegaly in VP16PPAR α mice. Most notably, there was a dramatic increase in the mortality of VP16PPAR α mice, suggesting that PPs might be of high toxicity to rodent liver as the basis of hepatomegaly.

The data from the VP16PPAR α mice suggest that in addition to hepatocytes, liver NPCs are critical in PP-induced tumorigenesis. By screening microarray data, genes involved in the DNA damage response network, especially in checkpoint signaling cascades, were dramatically induced both in wild-type and in VP16PPAR α liver upon Wy-14,643 challenge. Chek1 is a serine/threonine kinase that plays a critical role in cell survival and checkpoint regulation as a DNA damage-induced checkpoint regulator in enforcing cell cycle arrest (Dai and Grant, 2010). It is also responsible for the instability of Cdc25a during an unperturbed cell cycle (Liu *et al.*, 2006). Chek1 is involved in DNA repair by targeting repair kinases (e.g., Prkdc), which, together with Ku70-Ku80 (designed the

TABLE 2
Differential Regulation of Genes Related with DNA Damage and Cell Cycle by Wy-14,643 in Wild-Type (WT) and VP16PPAR α Mice

Accession number	WT	WT + Wy (folds)	VP16PPAR α (folds)	VP16PPAR α + Wy (folds)	Gene description
NM_011234	1	5.21	1.5	6.29	RAD51 homolog (Rad51)
NM_011159	1	4.64	1.04	3.96	Protein kinase, DNA activated, catalytic polypeptide (Prkdc)
NM_007691	1	4.28	1.57	6.83	Checkpoint kinase 1 homolog (Chek1)
NM_016756	1	3.96	2.84	2.64	Cyclin-dependent kinase2 (Cdk2)
NM_029766	1	3.80	1.47	5.05	Denticleless homolog (Dtl)
NM_008564	1	3.46	1.47	3.27	Minichromosome maintenance deficient 2 (Mcm2)
NM_025676	1	3.23	1.51	2.99	Mcm8
NM_008568	1	2.67	1.56	2.78	Mcm7

FIG. 4. Comparative expression of genes related with DNA damage repair after Wy-14,643 treatment in wild-type and VP16PPAR α mice. (A) qPCR analysis for RAD51, protein kinase, DNA activated, catalytic polypeptide (Prkdc), checkpoint kinase 1 homologue (Chek1), and minichromosome maintenance deficient 2 mitotin (Mcm2) after Wy-14,643 treatment for 5 days. ** p < 0.01 versus groups with control diet; note that no significant changes between untreated wild-type and VP16PPAR α mice or between Wy-14,643-treated wild-type and VP16PPAR α group. (B) qPCR analysis of RNA extracted from Wy-14,643-treated PPAR α -null mice or corresponding wild-type controls. ** p < 0.01 compared with control-diet groups or Wy-14,643-treated PPAR α -null group.

DNA-PK complex), are important for DSB repair (Goudelock *et al.*, 2003). Chek1-dependent phosphorylation of Rad51 is required for DNA damage-induced homologous recombination (Sorensen *et al.*, 2005). Moreover, Chek1 blocks a caspase2-dependent apoptotic response (Sidi *et al.*, 2008). The functions of Chek1 are consistent with putative mechanisms of PP-induced tumorigenesis: increased DNA synthesis, cell proliferation, and apoptosis suppression (Klaunig *et al.*, 2003). In the present study, at least three Chek1-related pathways were found upregulated in a PPAR α -dependent manner: Chek1-Mcm2-7-DNA replication checkpoint, Chek1-Prkdc (DNA-PK)-DSB (double-strain break) repair, and Chek1-Rad51-HRR, indicating that DNA damage repair network may be critical in the process of PP-induced liver tumors. These findings are consistent with published data, in which genes involved in DNA damage repair were induced by

fenofibrate in rats (Nishimura *et al.*, 2007) or by Wy-14,643 in mice (Woods *et al.*, 2007).

Although Chek1 and its downstream DNA damage repair network are induced following Wy-14,643 treatment, it is still unclear how PPAR α activation upon Wy-14,643 treatment initiates DNA damage repair. One possible mechanism is oxidative stress. Treatment of rodents with PPs induces expression of genes encoding enzymes involved in peroxisomal and mitochondrial fatty acid β -oxidation, which produces reactive oxygen species as a byproduct (Woods *et al.*, 2007; Yang *et al.*, 2007; Yeldandi *et al.*, 2000). Previous studies also reported that several oxidative stress-related genes were upregulated upon PP challenge, such as Txnip, Sod2, Gpx2, and Cat (Woods *et al.*, 2007; Yang *et al.*, 2007). Collectively, these studies revealed the involvement of oxidative stress in the multiple effects of PPs. Taken together, the current work demonstrates a critical role for Chek1 and its DNA damage repair network in PP-induced hepatocarcinomas as revealed by comparing the responses of VP16PPAR α transgenic mice and wild-type mice to Wy-14,643 challenge, thus providing new insight into understanding the tumorigenic effects of PPs.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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