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Role of peroxisome proliferator-activated receptor-α (PPARα) in bezafibrate-induced hepatocarcinogenesis and cholestasis

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Prolonged administration of peroxisome proliferators to rodents typically leads to hepatocarcinogenesis. Peroxisome proliferator-activated receptor-α (PPARα) is required to mediate alterations in PPARα target gene expression, repress apoptosis, enhance replicative DNA synthesis, oxidative stress to DNA and hepatocarcinogenesis induced by the relatively specific PPARα agonist, Wy-14,643. Interestingly, administration of the less specific PPARα agonist, bezafibrate, leads to a modest induction of PPARα target genes in the absence of PPARα expression. In these studies, the role of PPARα in modulating hepatocarcinogenesis induced by long-term feeding of 0.5% bezafibrate was examined in wild-type (+/+) and PPARα-null (−/−) mice. The average liver weight was significantly higher in (+/+) and (−/−) mice fed bezafibrate than controls, but this effect was considerably less in (−/−) mice as compared with similarly treated (+/+) mice. Increased levels of mRNA encoding cell cycle regulatory proteins and DNA repair enzymes were found in (+/+) mice fed bezafibrate, and this effect was not found in (−/−) mice. In mice fed bezafibrate for 1 year, preneoplastic foci, adenomas and a hepatocellular carcinoma were found in (+/+) mice, while only a single microscopic adenoma was found in one (−/−) mouse. This effect was observed in both Sv/129 and C57BL/6N strains of mice, although only preneoplastic foci were observed in the latter strain. Interestingly, hepatic cholestasis was observed in 100% of the bezafibrate-fed (−/−) mice, and this was accompanied by significantly elevated hepatic expression of mRNA encoding bile salt export pump and lower expression of mRNA encoding cytochrome P450 7A1, consistent with enhanced activation of the bile acid receptor, farnesoid X receptor. Results from these studies demonstrate that the PPARα is required to mediate hepatocarcinogenesis induced by bezafibrate, and that PPARα protects against potential cholestasis.

Abbreviations: ACO, acyl CoA oxidase; BSEP, bile salt export pump; CDK4, cyclin-dependent kinase-4; CYP4A, cytochrome P450 4A1; CYP7A1, cytochrome P450 7A1; FXR, farnesoid X receptor; GADPH, glyceraldehyde-3-phosphate dehydrogenase; PCNA, proliferating cellular nuclear antigen; PPRE, peroxisome proliferator responsive elements.

Introduction

Since its initial discovery in 1990 (1), the diverse roles for the peroxisome proliferator-activated receptor-α (PPARα) have been elucidated. In response to ligand activation, PPARα heterodimerizes with retinoid X receptor α (RXRα), and after recruitment of co-activators, modulates transcription of genes containing peroxisome proliferator responsive elements (PPREs) in their promoter sequence. PPARα can be activated by a broad class of chemicals, collectively known as peroxisome proliferators, that include phthalates, fatty acids, dehydroepiandrostenedione sulfate, trichloroethylene and the hypolipidemic fibrate drugs (e.g. bezafibrate, clofibrate, ciprofibrate, etc.). In recent years, it was determined that PPARα has a critical regulatory role in lipid homeostasis as its target genes include many enzymes and proteins involved in the transport and catabolism of fatty acids (2). However, while PPARα is essential for regulating lipid homeostasis, it is also known to mediate hepatocarcinogenesis typically observed in rodents fed peroxisome proliferators. This conclusion is based on the fact that PPARα-null mice do not exhibit hepatocarcinogenesis after long-term feeding of Wy-14,643 (3). Given the fact that the fibrate class of hypolipidemic drugs is still in use today, and that other chemicals known to act as PPARα agonists are present in the environment, there is concern about the relative risk for humans exposed to these agents.

The mode of action underlying PPARα agonist-induced hepatocarcinogenesis has not yet been fully elucidated. In response to ligand activation, PPARα mediates alterations in gene expression that lead to increased hepatic cell proliferation, decreased apoptosis and increased signaling for replicative DNA synthesis, that ultimately causes mutant cell populations to proliferate and become neoplastic (4). While PPARα is required to mediate Wy-14,643-induced hepatocarcinogenesis, the specific target genes modulated by this nuclear receptor that lead to discordant cell cycle regulation have not been identified. It is known that a number of proteins required for transition into the S-phase of the cell cycle are increased by peroxisome proliferators, and this event requires PPARα (5), although functional PPREs have not been characterized in any of these critical regulatory proteins. Additionally, oxidative stress induced by activated Kupffer cells in response to peroxisome proliferators may contribute to increased signaling for hepatocyte proliferation (6). Whether or not increased intracellular H_2O_2 resulting from elevated acyl CoA oxidase (ACO) expression in liver parenchymal cells contributes to oxidative stress-mediated signaling for increased cell proliferation is uncertain, but increased expression of ACO and resulting generation of H_2O_2 may lead to oxidative DNA damage (7). However, the evidence linking peroxisome proliferators to secondary oxidative DNA damage is conflicting, and therefore the mechanisms underlying the initiation of DNA damage in response to this class of chemicals have not been elucidated (8).

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The observation that PPARα-null mice are refractory to Wy-14,643-induced hepatocarcinogenesis and alterations in cell proliferation associated with the early events that probably contribute to the mechanisms of peroxisome proliferator-induced liver cancer (3,5), provides convincing evidence that PPARα is required to mediate this effect. However, the role of this receptor in peroxisome proliferator-induced hepatocarcinogenesis has only been examined using one relatively specific PPARα agonist, Wy-14,643. Interestingly, a recent report demonstrated that bezafibrate could induce the expression of a number of different PPARα target genes in PPARα-null mice. While bezafibrate markedly increased the hepatic expression of mRNAs encoding peroxisomal and mitochondrial fatty acid oxidizing enzymes in wild-type mice, a marginal increase in the same mRNAs was observed in the absence of PPARα expression (9). In contrast, these changes in mRNA markers are completely absent in PPARα-null mice fed Wy-14,643 (10). It is of interest to note that the EC_{50} for receptor activation is at least two orders of magnitude different for Wy-14,643 and bezafibrate with values of 0.63 and 90 μM for mouse PPARα being reported, respectively (11). Because the effect of feeding only one prototype peroxisome proliferator (Wy-14,643) to PPARα-null mice has been examined to date, the goal of the present studies was to examine the hypothesis that PPARα is required for bezafibrate-induced hepatocarcinogenesis using a peroxisome proliferator with inherently different PPARα activity. Additionally, a secondary goal of these studies was to examine the effect of marginally induced liver ACO on peroxisome proliferator-induced hepatocarcinogenesis, as the less specific PPARα agonist bezafibrate (as compared with Wy-14,643), is capable of inducing a PPARα target protein (ACO) in the absence of PPARα expression, and has been postulated to contribute to oxidative DNA damage during peroxisome proliferator-induced hepatocarcinogenesis.

Materials and methods

Diet

2-{4-[2-(4-Chlorobenzamido)ethyl]phenoxy}-2-methylpropanoic acid (bezafibrate) was purchased from Sigma Chemical (St Louis, MO). The pelleted mouse diet was prepared by Bioserv (Frenchtown, NJ) containing 0 (control) and 0.5% bezafibrate. Diets were available to mice *ad libitum.*

Short-term study

PPARα (+/+) and (−/−) male mice with an Sv/129 genetic background (10,12) were housed in a temperature (25°C) and light (12-h light/12-h dark) controlled environment. Mice were fed either the control or 0.5% bezafibrate diet for 1 or 5 weeks. Following this treatment, the mice were killed by overexposure of carbon dioxide after a 2-h fast. Body and liver weights were recorded, and the liver was snap-frozen in liquid nitrogen for subsequent analysis of mRNAs. A separate cohort of PPARα (+/+), PPARα (−/−), PPARα (−/−)/PPARβ (−/−) or PPARα (−/−)/PPARγ (−/−) [liver-specific deletion using albumin-Cre/floxed PPARγ alleles (13)] mice were fed 0.5% bezafibrate for 2 weeks, killed after a 2-h fast, and their liver collected and analyzed for mRNA as described above.

Long-term study

PPARα (+/+) and (−/−) mice with either Sv/129 or C57BL/6N genetic backgrounds were housed as described above. Mice from both genotypes were fed either the control or 0.5% bezafibrate diet for up to 12 months. One of the (−/−) mice, and three (+/+) mice fed bezafibrate died unexpectedly after 10 months of feeding, and another two (−/−) mice fed bezafibrate for 11 months were killed because of signs of morbidity. The livers from the four former mice were examined for grossly visible signs of liver tumors, but were not examined histologically due to autolysis. Tissues from the latter two (−/−) mice that were killed early were examined for grossly visible signs of liver tumors and included for additional histological analysis as described below. At the end of the 12-month feeding period, mice were killed after a 2-h fast; livers were removed and rinsed in phosphate-buffered saline, and weighed. Gross lesions were counted, the location and diameter were recorded, and representative samples were prepared for histological examination (see below).

Pathology

Each liver was examined for the presence of grossly visible lesions. For each mass, the diameter was measured and the location noted. Representative liver samples were removed and fixed in 10% phosphate-buffered formalin (Fisher Scientific, Fair Lawn, NJ) and embedded in paraffin. Paraffin sections were prepared from these samples, sections were stained with hematoxylin and eosin and examined morphologically for the presence of carcinomas, adenomas or preneoplastic lesions using established criteria (14). Control sections were also examined from mice lacking grossly visible lesions.

Northern blot analysis

Total RNA was isolated from liver sections lacking gross lesions by the Trizol method using the manufacturer's procedure (Invitrogen Life Technologies). Five to ten micrograms of RNA was separated by gel electrophoresis on 1% agarose gels containing 0.22 M formaldehyde, and transferred to nylon membrane in 10× SSC (1× SSC is 0.15 M NaCl and 0.18 M sodium citrate) overnight. Membranes were dried, UV cross-linked, washed twice in 2× SSC (1× SSC is 0.15 M NaCl and 0.18 M sodium citrate) and 0.5% sodium dodecyl sulfate for 15 min at 65°C. The following previously described cDNA probes were used for northern blotting: ACO, cytochrome P450 4A1 (CYP4A), cyclin-dependent kinase-4 (CDK4), proliferating cellular nuclear antigen (PCNA), cyclin B1, catalase, farnesoid X receptor (FXR), bile salt export pump (BSEP), cytochrome P450 7A1 (CYP7A1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control (5,10,15,16). Probes were labeled with [^{32}P]dCTP using a random priming method and hybridized in ULTRAhyb^{TM} buffer (Ambion, Austin, TX) at 42°C overnight, and washed. Quantification of hybridization signals was performed using phosphor imaging and expression data were normalized relative to the GAPDH signal. Representative liver RNA samples from between 3 and 4 mice were used for analysis per treatment group.

Hepatic bile acids

Frozen liver samples were homogenized in 75% ethanol, and incubated at 50°C for 2 h. After centrifugation, the supernatant was obtained and used for enzymatic determination of bile acids using the Sigma Diagnostics Bile Acid colorimetric assay (St Louis, MO).

Ribonuclease protection assays

Total RNA from frozen liver samples was isolated using RNeasy total RNA extraction kit (Qiagen, Valencia, CA) and dissolved in RNase-free water. Samples were stored at −80°C until assayed for no longer than 2 months to minimize degradation. The quality of preparations was determined using an Agilent Bio-Analyzer®. Expression of base excision DNA repair genes was determined using an RNase protection assay using mouse multi-probe RNA probe template set (mBER-1, BD PharMingen, San Diego, CA) as described previously (17). Riboprobes were synthesized in the presence of [^{32}P]dUTP to yield labeled antisense RNA probes. The RNase protection assays were performed using 30 μg of individual total RNA samples using a RiboQuant^{TM} multi-probe RNase protection assay kit. Protected fragments were separated on 5% polyacrylamide nucleic acid separation gels, dried and exposed to phosphor-imaging screen. The intensity of protected bands was quantified using a phosphor image analyzer and normalized to the intensity of housekeeping gene L32.

Statistical analysis

Differences between treatments were determined using ANOVA followed by Dunnett's post-hoc test (Prism 4.0, GraphPad Software, San Diego, CA). Significant differences were determined with $P \leq 0.05$.

Results

Consistent with previous work, administration of bezafibrate caused a large fold increase in the expression of mRNAs encoding ACO and CYP4A in the liver of (+/+) mice after 1 or 5 weeks as compared with the control. In contrast, induction of ACO and CYP4A was still observed in the liver of (−/−) mice fed bezafibrate as compared with similarly treated (+/+) mice but the fold induction was considerably lower (Figure 1A). Mice nullizygous for both PPARα and PPARβ, or PPARα and PPARγ were fed bezafibrate to determine if this

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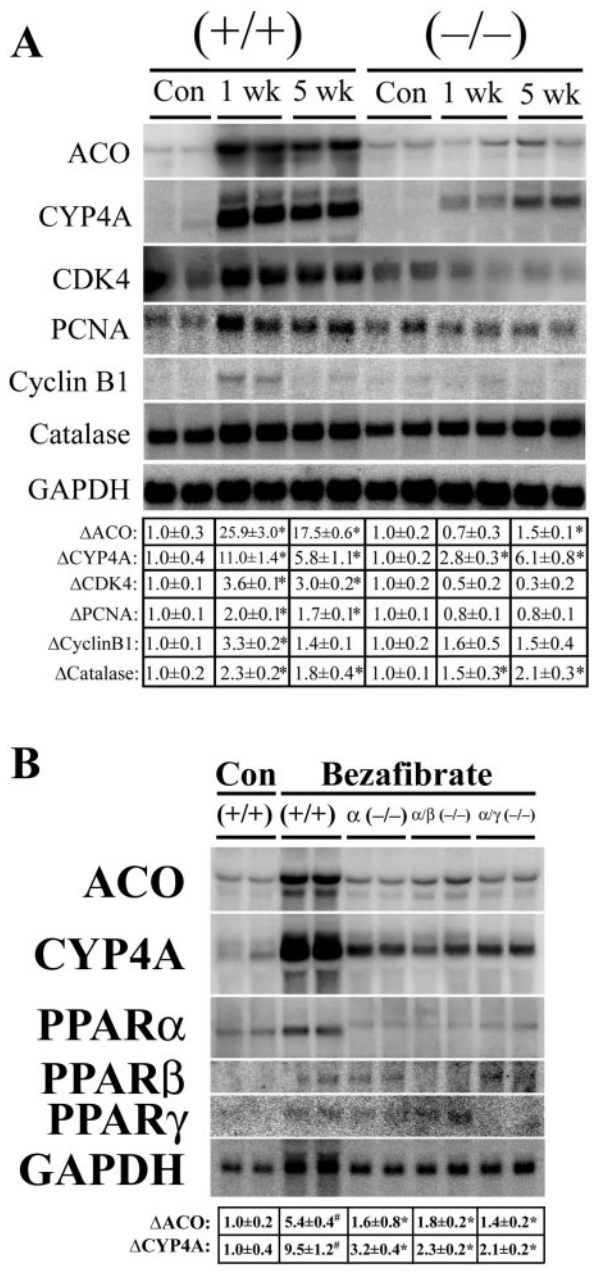

ΔACO:	1.0±0.3	25.9±3.0*	17.5±0.6*	1.0±0.2	0.7±0.3	1.5±0.1*
ΔCYP4A:	1.0±0.4	11.0±1.4*	5.8±1.1*	1.0±0.2	2.8±0.3*	6.1±0.8*
ΔCDK4:	1.0±0.1	3.6±0.1*	3.0±0.2*	1.0±0.2	0.5±0.2	0.3±0.2
ΔPCNA:	1.0±0.1	2.0±0.1*	1.7±0.1*	1.0±0.1	0.8±0.1	0.8±0.1
ΔCyclinB1:	1.0±0.1	3.3±0.2*	1.4±0.1	1.0±0.2	1.6±0.5	1.5±0.4
ΔCatalase:	1.0±0.2	2.3±0.2*	1.8±0.4*	1.0±0.1	1.5±0.3*	2.1±0.3*

ΔACO:	1.0±0.2	5.4±0.4#	1.6±0.8*	1.8±0.2*	1.4±0.2*
ΔCYP4A:	1.0±0.4	9.5±1.2#	3.2±0.4*	2.3±0.2*	2.1±0.2*

Fig. 1. Representative northern blot of hepatic mRNAs encoding ACO, CYP4A, catalase and cell cycle regulatory proteins, including, CDK4, PCNA and cyclin B1. (**A**) Total RNA was isolated from wild-type (+/+) and PPARα-null (−/−) mice with an Sv/129 genetic background, fed either a control diet or one containing 0.5% bezafibrate, for 1 (1 wk) or 5 weeks (5 wk). Total RNA was separated by gel electrophoresis, transferred to nylon membranes and probed with the indicated cDNAs. Representative samples from two independent samples per treatment are shown; a total of four independent samples were analyzed. Hybridization signals were normalized to GAPDH and are presented as the fold-increase as compared with respective control. *Significantly different than controls, $P \leq 0.05$. (**B**) Representative northern blot of hepatic mRNAs encoding ACO, CYP4A or peroxisome proliferator-activated receptors (PPARs) from control (+/+) or bezafibrate-fed (+/+), PPARα-null (−/−), PPARα/PPARβ-null [α/β (−/−)], or PPARα/PPARγ-null [α/γ (−/−)]. Note the absence of endogenous mRNA encoding PPARα, PPARβ or PPARγ as shown previously (10,13,35), in the (−/−), α/β (−/−) and α/γ (−/−) mice. Representative samples from two independent samples per treatment are shown; a total of at least three samples were analyzed. Hybridization signals were normalized to GAPDH and are presented as the fold-increase as compared with respective control. #Significantly different than wild-type control, $P \leq 0.05$. *Significantly different than wild-type control and bezafibrate-treated wild-type, $P \leq 0.05$.

marginal increase in ACO and CYP4A mRNA was mediated by either PPARβ and/or PPARγ. Consistent with previous studies demonstrating that PPARβ does not mediate bezafibrate-induced increases in liver ACO and CYP4A (9), mice nullizygous for both PPARα and PPARβ exhibited a marginal induction of mRNA encoding ACO and CYP4A (Figure 1B). Additionally, mice nullizygous for both PPARα and PPARγ exhibited a similar, marginal increase in both ACO and CYP4A mRNAs (Figure 1B). Bezafibrate feeding resulted in a similar increase in the mRNA encoding catalase in both (+/+) and (−/−) mice (Figure 1A). In contrast to the results observed with ACO and CYP4A expression, the level of mRNA encoding CDK4 and PCNA were significantly higher in (+/+) mice fed bezafibrate for 1 or 5 weeks, and the level of mRNA encoding cyclin B1 was significantly higher in this group as compared with (+/+) controls, but this effect was not found in similarly treated (−/−) mice (Figure 1A). This is consistent with the ~2-fold increase in average liver weight observed in (+/+) mice fed bezafibrate for 1 or 5 weeks (data not shown). Despite no changes in mRNAs encoding markers of cell cycle progression in the (−/−) mice fed bezafibrate for either 1 or 5 weeks, the average liver weight was marginally increased as compared with the control by ~1.5-fold (data not shown).

After 1 year of treatment with 0.5% bezafibrate in the diet, the average liver weight was ~4-fold higher in (+/+) mice than in the control (+/+) mice, while the average liver weight was only marginally increased (~2-fold) in (−/−) mice fed bezafibrate as compared with the control (−/−) mice (Table I). Grossly visible lesions consistent with the presence of adenomas or carcinomas were found in 100% of the (+/+) mice fed bezafibrate for 1 year whereas grossly visible lesions were not found in the (−/−) mice fed bezafibrate for the same timeframe (Table I). Average tumor multiplicity was 5 ± 2 lesions per liver in bezafibrate-fed (+/+) mice. Microscopic examination of liver sections was highly consistent with the gross examinations, as histological evidence of hepatic neoplasias was observed in 100% of the (+/+) mice fed bezafibrate for 1 year (Table II). The histological examination revealed that the majority of liver lesions were either adenomas or preneoplastic foci, but one well-differentiated carcinoma was observed in the (+/+) mice fed bezafibrate for 1 year (Figure 2). Interestingly, one adenoma was found in a (−/−) mouse fed bezafibrate (Figure 2), however, this was also accompanied by a unique phenotype not observed previously in response to long-term Wy-14,643 feeding. In addition to significant lipid accumulation (steatosis), liver pathology consistent with cholestasis was found in 100% of the (−/−) mice fed bezafibrate for 1 year that was not found in similarly treated (+/+) mice. Pigmented hepatocytes, consistent with bile acid accumulation, were noted in 100% of the (−/−) mice fed bezafibrate (Figure 2).

The presence of cholestasis in the (−/−) mice fed bezafibrate suggests an imbalance in bile acids, which was further evaluated by examining the concentration of hepatic bile acids and mRNAs encoding key regulators of bile acid homeostasis. Bezafibrate feeding resulted in a significant decrease in the concentration of hepatic bile acids in (+/+) mice (Figure 3A). A significant decrease in hepatic bile acid concentration was also observed in bezafibrate-fed (−/−) mice, but the percentage decrease was lower and the average concentration was significantly greater than that found in bezafibrate (+/+) mice (Figure 3A). To determine the biological significance

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Table I. Effect of bezafibrate on average relative liver weight and grossly visible hepatic lesions in wild-type (+/+) and PPARα-null (−/−) mice with an Sv/129 genetic background

Genotype	n[a]	Diet	Relative liver weight (% body wt)	Incidence[b]	Multiplicity[c]	Size[d] (mm)
(+/+)	8	Control	3.8 ± 0.2	0/8 (0%)	0 ± 0	0 ± 0
(+/+)	9	0.5% bezafibrate	16.3 ± 4.0[†]	7/7[*] (100%)	5.3 ± 2.1[*]	4.0 ± 0.7[*]
(−/−)	9	Control	5.1 ± 0.9	0/9 (0%)	0 ± 0	0 ± 0
(−/−)	9	0.5% bezafibrate	7.8 ± 0.8[†]	0/9 (0%)	0 ± 0	0 ± 0

Mice with an Sv/129 genetic background were fed respective diet for up to 1 year and examined for liver lesions upon dissection. Three (+/+) mice died after ~10 months of feeding and their analysis is included in the data. Data from one (−/−) mice that died after ~10 months of feeding, and two (−/−) mice that were killed after 11 months of feeding are included as well.
[a]The number of mice examined.
[b]The incidence of mice with grossly visible liver lesions. The percentage of mice is in parentheses.
[c]The average number of grossly visible liver lesions per mouse.
[d]The average size of grossly visible liver lesions per mouse.
[*]Significantly greater than controls, $P \leq 0.05$.
[†]Signficantly different than the control and bezafibrate-fed (+/+) mice, $P \leq 0.05$.

Table II. Effect of bezafibrate on the incidence of hepatocellular neoplasms in the wild-type (+/+) and PPARα-null (−/−) mice with an Sv/129 genetic background

Genotype	n[a]	Diet	Number of livers with indicated hepatocellular lesion				
			Peroxisome proliferation[b]	Multiple foci[c]	Single adenoma	Multiple adenoma	Single carcinoma
(+/+)	4	Control	0/4	0/4	0/4	0/4	0/4
(+/+)	6	0.5% bezafibrate	6/6	6/6	2/6	2/6	1/6
(−/−)	4	Control	0/4	0/4	0/4	0/4	0/4
(−/−)	8	0.5% bezafibrate	0/8	0/8	1/8	0/8	0/8

Mice with an Sv/129 genetic background were fed the respective diet for up to 1 year and examined microscopically for liver lesions after H&E staining. Data from two (−/−) mice that were killed after 11 months of feeding are included as well.
[a]The number of livers examined.
[b]Histological evidence of peroxisome proliferation. Values represent the number of samples exhibiting peroxisome proliferation of the number of samples examined.
[c]Foci of cellular alterations.

of this difference in bile acid concentration, mRNA markers of bile acid homeostasis were examined. No difference in the expression pattern of mRNA encoding FXR was found between genotypes or treatment (Figure 3B). However, the expression of mRNA encoding BSEP that is known to be increased as a result of bile acid activation of FXR was significantly increased in the (−/−) mice after 1 week, 5 weeks and 1 year of feeding the bezafibrate diet (Figure 3B). In contrast, expression of mRNA encoding BSEP was reduced in (+/+) mice fed bezafibrate at similar time points (Figure 3B). Further, the expression of CYP7A1, which is known to be down-regulated by FXR was significantly lower at earlier time points in the bezafibrate-fed (−/−) mice as compared with similarly treated (+/+) mice (Figure 3B). These results are consistent with enhanced bile acid/FXR-mediated signaling in the (−/−) mice fed bezafibrate.

As hepatic ACO mRNA expression was marginally increased in the (−/−) fed bezafibrate for 5 weeks, an RNase protection assay was performed to examine the relative expression of mRNA markers for oxidative stress to DNA. In (+/+) mice, bezafibrate treatment led to a significant time-dependent increase in expression of mRNAs encoding 8-oxoguanine DNA glycosylase/lyase 1 (*Ogg1*), apurinic/apyrimidinic endonuclease 1 (*Ape*), O^6-methyl guanine methyltransferase (*Mgmt*), *N*-methylpurine DNA glycosylase (*Mpg*), thymine DNA glycosylase (*Tdg*) and endonuclease III homolog 1 (*Nth1*) (Figure 4). However, expression of mRNAs encoding DNA repair proteins was not affected in the bezafibrate-treated (−/−) mice (Figure 4).

All of the previous work examining the role of PPARα in mediating peroxisome proliferator-induced hepatocarcinogenesis, including these studies and prior results (3), have utilized mice with an Sv/129 genetic background. In these studies, a small cohort of mice with the C57BL/6N genetic background were also examined after 1 year of feeding 0.5% bezafibrate. Hepatomegaly was found in (+/+) mice with a C57BL/6N genetic background fed bezafibrate for 1 year, and similar to results observed in the Sv/129 mice, a small increase in average liver weight was also found in (−/−) mice with a C57BL/6N genetic background fed bezafibrate for 1 year (Table III). In contrast to the high incidence of grossly visible lesions found in the Sv/129 (+/+) mice fed bezafibrate for 1 year, no grossly visible lesions were observed in the bezafibrate-fed (+/+) mice with a C57BL/6N genetic background, nor in the (−/−) mice on the same genetic background (Table III). While no adenomas or carcinomas were found in the C57BL/6N (+/+) mice fed bezafibrate, microscopic examination revealed preneoplastic foci in 75% of the livers examined (Table IV). No microscopic evidence of neoplasias was found in any of the (−/−) mice with the C57BL/6N genetic background fed bezafibrate for 1 year (Table IV), although cholestasis was noted in all of these samples in the absence of significant lipid accumulation (Figure 2).

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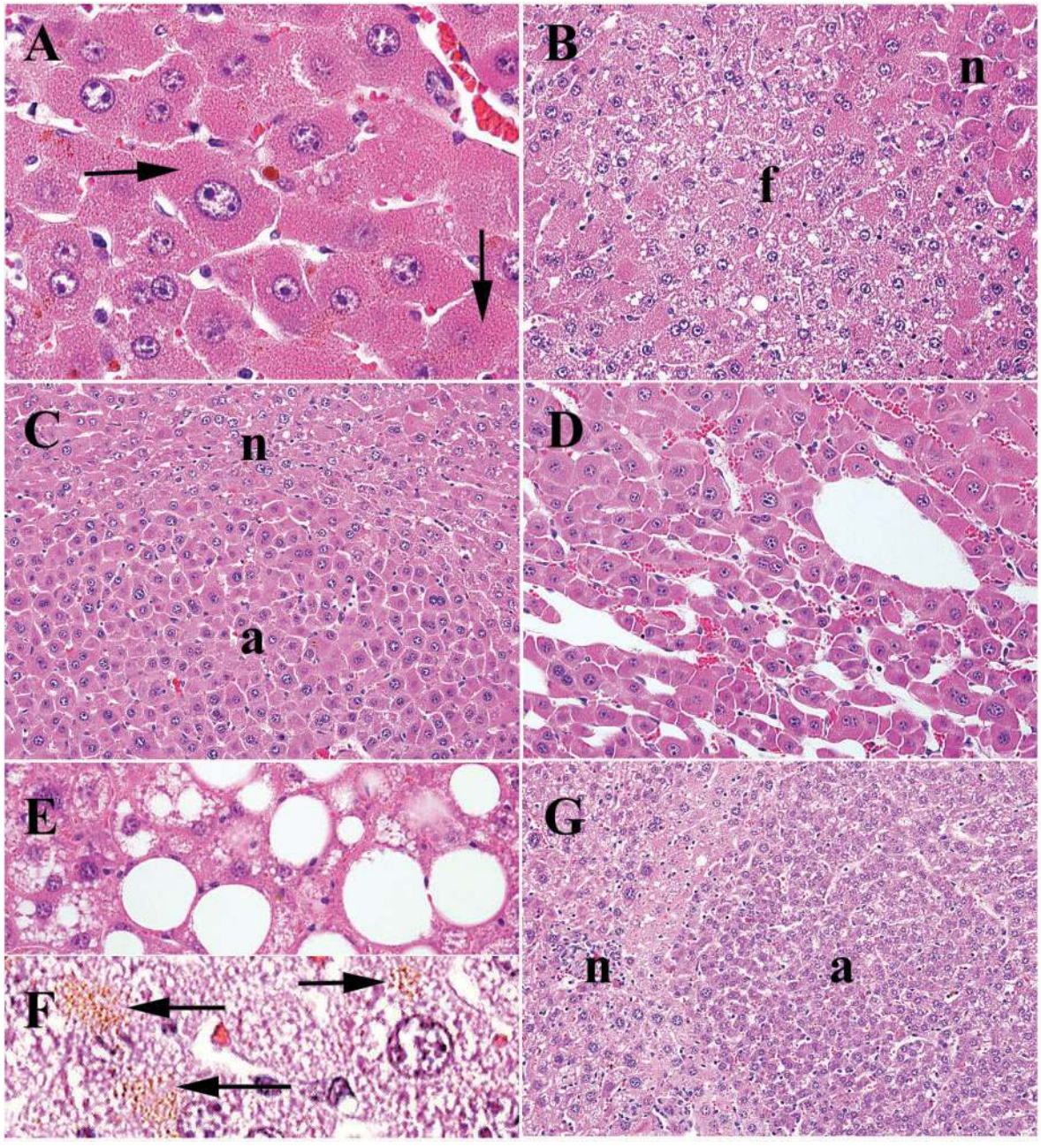

Fig. 2. Representative histopathology of the liver in response to bezafibrate treatment. Wild-type (+/+) or PPARα-null (−/−) mice were fed either a control or 0.5% bezafibrate diet for 1 year, and livers examined. (**A**) Representative photomicrograph of peroxisome proliferation in (+/+) mouse liver. This phenotype was observed in both (+/+) Sv/129 and (+/+) C57BL/6N mouse liver, but not in the (−/−) liver from either strain. Note the dense presence of peroxisomes (eosinophilic cytoplasmic granularity) in the hepatocyte cytosol (arrows). Magnification = 400×. (**B**) Representative photomicrograph of preneoplastic irregular clear cell focus in the (+/+) mouse liver. The focus is indicated by 'f' and the normal tissue surrounding the focus is indicated by 'n'. These lesions were observed in both (+/+) Sv/129 and (+/+) C57BL/6N mouse liver, but not in the (−/−) liver from either strain. Magnification = 200×. (**C**) Representative photomicrograph of hepatocellular adenoma from the (+/+) mouse. The adenoma and adjacent liver are both eosinophilic. The adenoma is indicated by 'a' and the surrounding normal tissue is indicated by 'n'. Magnification = 200×. (**D**) Representative photomicrograph of well-differentiated hepatocellular carcinoma from the (+/+) mouse. Magnification = 400×. (**E**) Representative photomicrograph of lipid accumulation observed in the (−/−) mice fed bezafibrate with an Sv/129 genetic background. Magnification = 400×. (**F**) Representative photomicrograph of cholestasis present in 100% of the (−/−) mice, both strains. Arrows indicate the presence of pale, brown finely granular pigment in the cytoplasm of hepatocytes. Magnification = 400×. (**G**) Photomicrograph of the hepatocellular adenoma from the one (−/−) mouse. The adenoma is basophilic and the adjacent liver is much less eosinphilic than the (+/+) mice fed bezafibrate. The adenoma is indicated by 'a' and the surrounding normal tissue is indicated by 'n'. Magnification = 200×.

Discussion

Results from these studies show that the wild-type mice fed bezafibrate exhibit many of the hallmark changes associated with activation of PPARα including induction of ACO and CYP4A, induction of mRNAs encoding cell cycle regulatory proteins and DNA repair enzymes, hepatomegaly, and the resulting high incidence of hepatocellular neoplasms. While the hepatocarcinogenic effects of long-term bezafibrate feeding have not been reported in mice, it is well established that long-term feeding of other fibrates and PPARα agonists to

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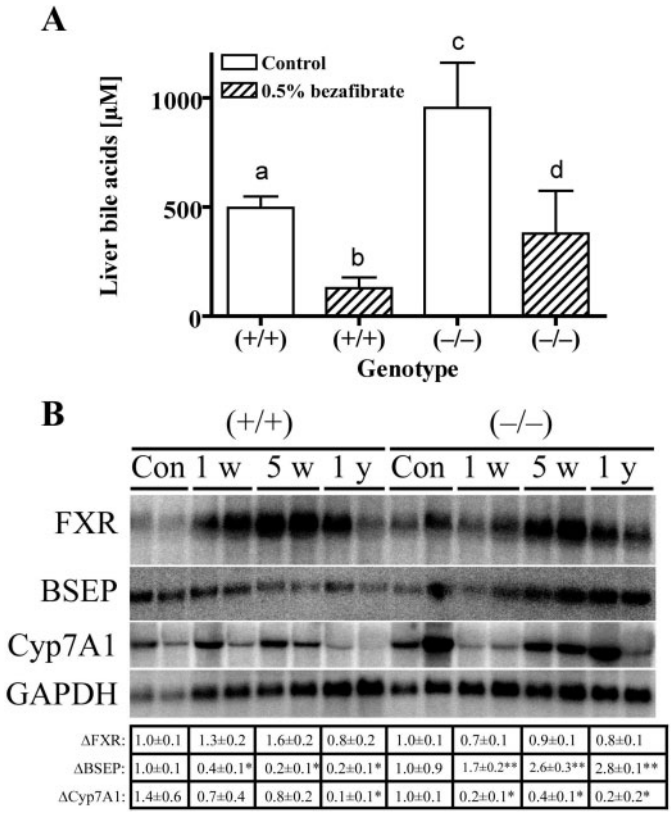

Fig. 3. Hepatic concentrations of bile acids and representative northern blot of hepatic mRNAs encoding genes regulating bile acid homeostasis, including FXR, BSEP and CYP7A1. (**A**) The concentration of bile acids were measured as described in the liver from mice fed bezafibrate for 1 year. Values with different letters are significantly different, $P \leq 0.05$. (**B**) Total RNA was isolated from the liver, separated by gel electrophoresis, transferred to nylon membranes and probed with the indicated cDNAs as described in the Materials and methods. Representative samples are shown from wild-type (+/+) and PPARα-null (−/−) mice fed either a control diet or a diet containing 0.5% bezafibrate for 1 week (1 w), 5 weeks (5 w) or 1 year (1 y). Hybridization signals were normalized to GAPDH and presented as a fold increase as compared with respective control. A total of four independent samples were analyzed per treatment group, with representative results from two independent samples depicted. *Significantly lower than respective control, $P \leq 0.05$. **Significantly greater than respective control, $P \leq 0.05$.

rodents results in liver hepatocarcinogenesis (reviewed in ref. 4). Previous studies have demonstrated that the hepatocarcinogenic effect of a prototypical PPARα agonist, Wy-14,643, is mediated by PPARα, as PPARα-null mice are refractory to PPARα-induced changes including induction of cell cycle regulatory proteins (5), oxidative stress to DNA (18), and more importantly, hepatocellular carcinogenesis (3). Results from present studies that used the less specific PPARα agonist, bezafibrate, are relatively consistent with results from the former study. For example, no changes in hepatic levels of mRNAs encoding CDK4, PCNA and cyclin B1 or DNA repair enzymes were observed in bezafibrate-fed PPARα-null mice, and the incidence of hepatocellular neoplasms was significantly lower in the bezafibrate-fed PPARα-null mice as compared with similarly treated wild-type mice. However, in contrast to the previous studies demonstrating the lack of liver carcinogenesis in Wy-14,643-fed PPARα-null mice, one adenoma was found in a PPARα-null fed bezafibrate for 1 year. While this result suggests that PPARα may not be essential for mediating the hepatocarcinogenic effect of bezafibrate, the collective evidence from the present studies are inconsistent with this hypothesis, as no changes in markers of hepatocyte cell proliferation (e.g. CDK4, cyclin B1 and PCNA mRNAs) and oxidative DNA damage were found in bezafibrate-fed PPARα-null mice. As these changes are causally linked to the mode of action for peroxisome proliferator-induced hepatocarcinogenesis (4), the absence of these changes suggests that other mechanisms probably underlie the presence of the single adenoma in the PPARα-null mouse fed bezafibrate for 1 year. There are a number of possible variables that could account for this observation, including the presence of a rare, spontaneous neoplasm in an older mouse, differences in receptor/ligand affinity and the presence of altered bile acid homeostasis observed in the PPARα-null mice fed bezafibrate.

It is well documented that reporter constructs containing ACO PPREs (a PPRE from an endogenous PPARα-regulated target gene) can be activated by all three PPARs when treated with specific ligands (19). There is also some promiscuity associated with ligand and receptor specificity as PPARα ligands can also activate ACO PPREs when co-transfected with either PPARβ or PPARγ expression vectors (20–22). This ligand/receptor promiscuity also occurs with PPARβ and PPARγ ligands. Therefore, it is possible that the biological effects induced by 'specific' PPAR ligands could be mediated by more than one PPAR. The EC_{50} for mouse PPAR activation by Wy-14,643 based on cell-based transactivation assays is 0.63, >100 and 32 μM, while the EC_{50} for mouse PPAR activation by bezafibrate is 90, 110 and 55 μM, for mouse PPARα, PPARβ(δ) and PPARγ, respectively (11). Given the known ability for PPARα ligands to activate more than one PPAR, the fact that all three PPARs can transactivate similar response elements, and that the relative affinity for bezafibrate for PPARα is less specific as compared with Wy-14,643, suggests that bezafibrate-induced changes in gene expression could be mediated by either PPARβ or PPARγ. This is of particular interest because in the absence of PPARα expression, known PPARα target genes are still marginally induced by bezafibrate (9). The present studies indicate that this could be due to either PPARβ or PPARγ as a marginal induction of ACO and CYP4A is observed in the absence of both PPARα and PPARγ, or PPARα and PPARβ expression in the liver. This suggests that PPARβ or PPARγ could mediate this effect, but this hypothesis will require examination of mice nullizygous for all three PPARs. These combined observations suggest that the presence of an adenoma in the PPARα-null mouse fed bezafibrate could be the result of a marginal induction of PPARα target genes; however, results from the present studies are inconsistent with this hypothesis as no changes in mRNAs encoding cell cycle regulatory proteins or DNA repair enzymes were found in the PPARα-null mice fed bezafibrate. While the marginal induction of ACO and CYP4A observed in the PPARα-null mice fed bezafibrate could be due to activation of PPARβ or PPARγ [and increased expression of PPARγ is reported to occur in PPARα-null mice (23)], it is unlikely that this is causally related to the one adenoma observed in the PPARα-null mouse, as similar marginal induction of ACO that occurs with dietary modulation has never been shown to lead to hepatocarcinogenesis. Further, the lack of increased markers of oxidative DNA damage suggests that the marginal induction of ACO by bezafibrate in PPARα-null mice probably does not lead to DNA damage. As ligand activation of PPARγ is reported to cause hepatotoxicity (24), it is possible

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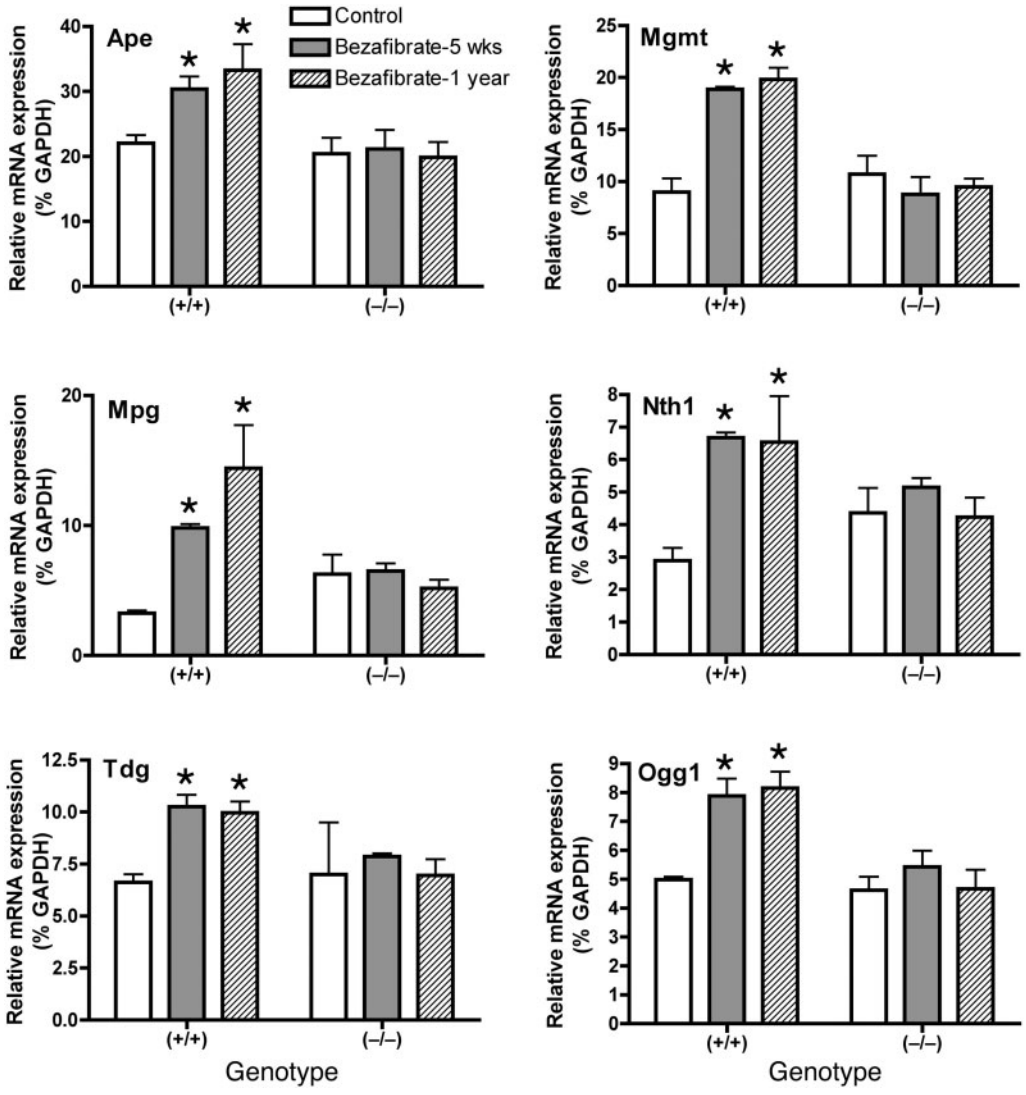

Fig. 4. Effect of bezafibrate on the expression of hepatic mRNAs encoding DNA repair proteins in wild-type (+/+) and PPARα-null (−/−) mice with an Sv/129 genetic background. Total RNA was isolated from (+/+) and (−/−) mice fed either the control or 0.5% bezafibrate diet for 5 weeks (5 wks) or 1 year, and an RNase protection assay performed as described in the Materials and methods. Note the increase in the expression of mRNAs encoding DNA repair proteins *Ogg1*, *Ape*, *Mgmt*, *Mpg*, *Tdg* and *Nth1*, and the lack of increase in the bezafibrate-treated (−/−) mice. Hybridization signals were normalized to GAPDH and are presented as a percentage of GAPDH. N = three independent RNA samples per treatment group. *Significantly different than controls, $P \leq 0.05$.

Table III. The effect of bezafibrate on average relative liver weight and grossly visible hepatic lesions in wild-type (+/+) and PPARα-null (−/−) mice with a C57BL/6N genetic background

Genotype	n[a]	Diet	Relative liver weight (% body wt)	Incidence[b]
(+/+)	4	Control	4.5 ± 2.2	0/4 (0%)
(+/+)	4	0.5% bezafibrate	12.4 ± 0.9*	0/4 (0%)
(−/−)	3	Control	4.7 ± 0.5	0/3 (0%)
(−/−)	5	0.5% bezafibrate	6.4 ± 1.3†	0/5 (0%)

Mice with a C57BL/6N genetic background were fed a respective diet for 1 year and examined for liver lesions upon dissection.
[a]The number of mice examined.
[b]The incidence of mice with grossly visible liver lesions. The percentage of mice is in parentheses.
*Significantly greater than controls, $P \leq 0.05$.
†Significantly different than the control and the bezafibrate-fed (+/+) mice, $P \leq 0.05$.

that the adenoma found in the bezafibrate-fed PPARα-null mouse resulted from activation of this PPAR isoform. The possibility that bezafibrate activates PPARγ in the PPARα-null mice is also supported by the observed steatosis in PPARα-null mice, since it is known that PPARγ is required for lipid accumulation in liver (25).

The presence of cholestasis in 100% of the PPARα-null mice fed bezafibrate indicates that the concentration of bile acids is considerably higher in these mice than similarly treated wild-type mice. While bezafibrate feeding resulted in a significant reduction in hepatic bile acid concentration in wild-type mice, the percentage decrease and the average concentration of hepatic bile acids were significantly greater in PPARα-null mice. These observations are consistent with the detected changes in gene expression of BSEP and CYP7A1 in the PPARα-null mice fed bezafibrate. Interestingly, these genes are known to be regulated by FXR, the nuclear receptor

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Table IV. Effect of bezafibrate on the incidence of hepatocellular neoplasms in wild-type (+/+) and PPARα-null (−/−) mice with a C57BL/6N genetic background

Genotype	*n*[a]	Diet	Number of livers with indicated hepatocellular lesion				
			Peroxisome proliferation[b]	Multiple foci[c]	Single adenoma	Multiple adenoma	Single carcinoma
(+/+)	4	Control	0/4	0/4	0/4	0/4	0/4
(+/+)	4	0.5% bezafibrate	4/4	3.4	0/4	0/4	0/4
(−/−)	3	Control	0/3	0/3	0/3	0/3	0/3
(−/−)	5	0.5% bezafibrate	0/5	0/5	0/5	0/5	0/5

Mice with a C57BL/6N genetic background were fed a respective diet for 1 year and examined microscopically for liver lesions after H&E staining.
[a]The number of livers examined.
[b]Histological evidence of peroxisome proliferation. Values represent the number of samples exhibiting peroxisome proliferation of the number of samples examined.
[c]Foci of cellular alterations.

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that controls hepatic bile acid homeostasis (16). Others have demonstrated previously that bile acids can function as tumor promoters (26,27), suggesting that a bezafibrate-induced increase in bile acids in the PPARα-null mice could promote tumor formation. Others have also shown that PPARα agonists, including Wy-14,643 and ciprofibrate, can inhibit bile acid synthesis (28), which is consistent with the observed increase in cholestasis found in PPARα-null mice fed bezafibrate. There is also evidence that conjugation of bile acids can be modulated by PPARα (29), thus, the absence of this regulation could lead to accumulation of conjugated bile acids that may contribute to the observed phenotype. However, it is important to point out that cholestasis is not observed in Wy-14,643-fed PPARα-null mice after long-term feeding (3), which suggests that this putative mechanism is probably not functional for all PPARα agonists and could be related to the differences in receptor affinity described above. It is also important to note that these alterations in bile acid homeostasis are not found in bezafibrate-fed wild-type mice that express a functional PPARα, suggesting that with this low affinity ligand, PPARα actually protects against cholestasis. The possibility remains that bezafibrate or one of its metabolites may actually be an FXR agonist. It is also of interest to note that bile acids (natural FXR ligands) antagonize PPARα agonist-induced modulation of target gene expression in liver indicating the possibility of cross-talk between PPARα and FXR (30). This observation suggests that in the absence of PPARα expression, bile acids could possibly activate FXR with greater efficiency. It will be interesting to determine whether other lower affinity fibrate drugs produce cholestasis in the absence of PPARα expression. Combined, these observations suggest that there is enhanced accumulation of bile acids and activation of FXR that would not normally occur in the presence of a functional PPARα, indicating a protective role for this nuclear receptor in preventing cholestasis in response to bezafibrate. This is of great interest because cholestasis is uncommon in mice, but is often observed in humans in response to therapeutic drugs (31).

An interesting strain difference in response to bezafibrate was also determined from these studies. Preneoplastic lesions were found in a high percentage (75%) of bezafibrate-fed wild-type mice with the C57BL/6N genetic background, but no adenomas or carcinomas were observed in these mice suggesting that in this genetic background, hepatocellular tumors develop more slowly than in the Sv/129 background. No evidence of neoplasia was found in bezafibrate-fed PPARα-null mice in the C57BL/6N genetic background, despite the presence of cholestasis (but no steatosis) in all of these mice. C57BL/6N mice fed the potent PPARα agonist ciprofibrate for 21 months exhibited an approximate incidence of 57% of hepatic neoplasia, while similar treatment with ciprofibrate for 18 months resulted in an approximate incidence of 62% (32). In contrast, all other published reports examining peroxisome proliferator-induced hepatocarcinogenesis have used mouse strains other than C57BL/6N, and a very high incidence of neoplasia ($>90\%$) is typically observed in these lines, dependent on the duration of exposure, dose and potency of PPARα agonist (reviewed in ref. 4). As the C57BL/6 strain of mouse is more resistant to hepatocarcinogenesis-induced peroxisome proliferators (32), and other chemicals (33,34), results from the present studies examining bezafibrate-induced hepatocarcinogenesis in C57BL/6N mice provide additional evidence that illustrates that the PPARα-null in an Sv/129 genetic background are more appropriate for examination of PPARα agonist-induced hepatocarcinogenesis. Additionally, the lack of preneoplastic foci in the PPARα-null mice with the C57BL/6N genetic background suggests that PPARα is required for bezafibrate-induced hepatocarcinogenesis in this mouse strain.

In summary, the extent of bezafibrate-induced changes in hepatic expression of mRNAs encoding ACO, cell cycle regulatory proteins and DNA repair enzymes, hepatomegaly, and the incidence of hepatocellular neoplasms is either completely lacking or significantly lower in PPARα-null mice as compared with similarly treated wild-type mice. These results are consistent with a previous study demonstrating the lack of these similar changes in PPARα-null mice fed Wy-14,643 for 11 months. This suggests that PPARα is essential for bezafibrate-induced hepatocarcinogenesis. The distinctly unique presence of altered bile acid homeostasis in PPARα-null mice treated with this relatively less specific PPARα agonist, coupled with the known tumor-promoting capabilities of bile acids, suggests that these alterations probably underlie the mechanisms leading to a minor incidence of neoplasia in the absence of PPARα expression. As the role of PPARα in peroxisome proliferator-induced hepatocarcinogenesis had previously only been examined using the prototypical peroxisome proliferator Wy-14,643, results from the present studies provide a new data set that strengthens the argument that PPARα is essential to mediate peroxisome proliferator-induced hepatocarcinogenesis.

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