

Activation of Peroxisome Proliferator–Activated Receptor Alpha Enhances Apoptosis in the Mouse Liver

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Received December 18, 2005; accepted April 13, 2006

Chronic exposure to peroxisome proliferators (PPs) leads to increased incidence of liver tumors in rodents. Liver tumor induction is thought to require increased hepatocyte proliferation and suppression of apoptosis. Transcript profiling showed increased expression of proapoptotic genes and decreased expression of antiapoptotic genes in the livers of mice exposed to the PP WY-14,643 (WY). We tested the hypothesis that prior exposure to WY would increase susceptibility to apoptosis inducers such as Jo2, an antibody which activates the Fas (Apo-1/CD95) death pathway. When compared to their untreated counterparts, wild-type mice pretreated with WY exhibited increased caspase-3 activation and hepatocyte apoptosis following challenge with Jo2. Livers from WY-treated peroxisome proliferator–activated receptor alpha (PPAR α)-null mice were resistant to the effects of Jo2. In the absence of Jo2 and detectable apoptosis, wild-type mice treated with WY exhibited increases in the activated form of caspase-9. As caspase-9 is a component of the apoptosome, we examined the expression of upstream effectors of apoptosome activity including members of the Bcl-2 family. The levels of the antiapoptotic Mcl-1 transcript and protein were significantly decreased by PPs. PPAR α -null mice were also resistant to another treatment (concanavalin A) that induces hepatocyte apoptosis. These results (1) indicate that PPAR α activation increases sensitivity of the liver to apoptosis and (2) identify a mechanism by which PPAR α could serve as a pharmacological target in diseases where apoptosis is a contributing feature.

Key Words: peroxisome proliferators; liver tumor; hepatocyte proliferation; apoptosis; caspase.

Peroxisome proliferators (PPs) are a large class of structurally heterogeneous industrial and pharmaceutical chemicals (Corton *et al.*, 2000). The nuclear receptor, peroxisome proliferator–activated receptor alpha (PPAR α), mediates many, if not all, of the adaptive consequences of PP exposure in the liver including alteration in lipid metabolism genes, hepatomegaly, and increases in liver tumors (Corton *et al.*, 2000; Klaunig *et al.*, 2003; Lee *et al.*, 1995; Peters *et al.*, 1997). PP exposure increases the size and number of hepatocytes within the first few days of exposure. During this time, spontaneous hepatocyte apoptosis is suppressed within the intact liver (James *et al.*, 1998). Rodent primary hepatocytes successfully recapitulate these early responses to PP exposure observed *in vivo* and have been used to demonstrate that after PP exposure, suppression of spontaneous apoptosis as well as apoptosis induced by TGF β , DNA damage, and Fas (Apo-1/CD95) is PPAR α dependent (Gill *et al.*, 1998; Hassmall *et al.*, 2000). The results of the primary hepatocyte work support the hypothesis that suppression of apoptosis contributes to PP-induced liver tumor formation in rodents (James *et al.*, 1998).

Apoptosis is carried out by caspases, a family of proteases that sequentially disassemble a cell (Hengartner, 2000; Wang, 2001). The pathways leading to caspase activation are dependent on the cytotoxic stimulus. Cytotoxic stress including DNA damage activates caspases by initiating signaling pathways that converge on the Bcl-2 family of proteins (Gross *et al.*, 1999). After apoptotic stimulation, changes in the balance between pro- and antiapoptotic members of this family lead to alteration of mitochondrial pore structure or integrity and permeabilization and release of proteins that promote cell death (Huang and Strasser, 2000). One of these released proteins is cytochrome c which forms a supramolecular complex called the apoptosome with the cytoplasmic protein Apaf-1, leading to activation of caspase-9 (Adrain and Martin, 2001). Caspase-9 in turn activates the effector caspase, caspase-3 which cleaves many of the caspase substrates during apoptosis.

In the present study, we examined the expression of genes in mouse liver after exposure to the PP WY-14,643 (WY) known

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to be involved in apoptosis and are found on the platform that we previously used to query gene expression (Anderson *et al.*, 2004a,b). The overall gene expression pattern indicated there was a general upregulation of proapoptotic genes and downregulation of antiapoptotic genes. Based on these results, we tested the hypothesis that livers from wild-type mice treated with a PP exhibit increased susceptibility to apoptosis inducers compared to PPAR α -null mice.

MATERIALS AND METHODS

Animals. All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the CIIT Centers for Health Research. Details of animal husbandry used in our studies have been described (Fan *et al.*, 2003). Male wild-type and PPAR α -null mice (9 \pm 1 week) were treated with different chemicals in four separate studies: (1) mice were given WY (ChemSyn Science Laboratories, Lenexa, KS; 50 mg/kg) in 0.1% methylcellulose or 0.1% methylcellulose alone by gavage each day for 3 days, (2) mice were fed a diet containing 0.05% WY for 1 week, (3) trichloroacetate (TCA) (Sigma Chemical Co, St Louis, MO; 2 g/l) dissolved in the drinking water and adjusted to a pH 7.0–7.2 was given to mice for 1 week, and (4) the pan-specific RXR agonist AGN-194,204 (Allergan, Irvine, CA) (3 mg/kg/day) in 0.1% methylcellulose or 0.1% methylcellulose alone was given to mice by daily gavage each day for 3 days.

Induction of apoptosis. Mice were challenged with two different apoptosis inducers in these studies. After pretreatment for 1 week with the WY-containing diet or a control diet, mice were given one iv injection of Jo2 antibody (PharMingen, San Diego, CA; 6 μ g/kg body weight) or one iv injection of concanavalin A (Sigma Chemical Co; 25 mg/kg). Mice were sacrificed at the indicated times or sacrificed when moribund (Jo2 study only). Preparation of the livers for analysis of molecular endpoints was carried out as described (Fan *et al.*, 2003).

Assessment of apoptosis. Apoptosis in liver tissues was detected by measuring the appearance of apoptotic bodies with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using the ApopTag peroxidase kit (Invitrogen, Carlsbad, CA) and by pathological quantitation of apoptotic foci. Approximately 3000 average cells were counted per sample.

RNA isolation and analysis of gene expression by gene arrays. Three mice were analyzed from each treatment group, for a total of 12 analyses as previously described (Anderson *et al.*, 2004a,b). The groups were control wild type, WY-treated wild type, control PPAR α -null, and WY-treated PPAR α -null. Hepatic RNA was isolated using a modified guanidium isothiocyanate method (TRIzol, Invitrogen) and was further purified using silica membrane spin columns (RNEasy Total RNA Kit, Qiagen, Valencia, CA). RNA integrity was assessed by ethidium bromide staining followed by resolution on denaturing agarose gels and also by the RNA 6000 LabChip Kit using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). For each sample, 15 μ g of biotin-labeled cRNA was generated from 10 μ g total RNA and hybridized to Murine GeneChip U74Av2 oligonucleotide arrays (Affymetrix, Mountainview, CA). All procedures were carried out according to manufacturer's recommendations using the antibody amplification technique. Images were initially processed using the MAS 5.0 software (Affymetrix). Hybridization quality was assessed by visual inspection of the image and from a report generated by MAS 5.0. The data were analyzed and statistically filtered using Rosetta Resolver version 3.0 software (Rosetta Inpharmatics, Kirkland, WA). The threshold for significance was set at $p \leq 0.001$, and the relative increase or decrease in mRNA abundance for each gene was reported as a fold change relative to the control. Genes were grouped into functional classes with the help of KEGG (<http://www.kegg.org>)

and Gene Ontology (<http://www.geneontology.org>). Identification of expressed sequence tags was facilitated by euGenes (<http://iubio.bio.indiana.edu:89/mouse/>). Genes were clustered and visualized using CLUSTER and TreeView (Eisen *et al.*, 1998).

Analysis of protein expression. Preparation of liver lysates and Western blot analyses were as previously described (Fan *et al.*, 2003). Proteins were transferred to nitrocellulose (Stratagene, La Jolla, CA) and visualized by Ponceau Red to confirm transfer and equal loading of the lanes. To further confirm equal loading, we assessed the expression of a number of common "housekeeping" proteins including tubulin and actin in the same extracts. These were found to be inappropriate as expression was altered after exposure to WY as determined by transcript profiling and Western blots (data not shown). Instead, we used HSP65 expression as a loading control as expression does not detectably change after exposure to PPC. Immunoblots were developed using polyclonal anti-caspase-3, anti-caspase-8 (Cell Signaling Technology, Danvers, MA), anti-caspase-9 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HSP65 (StressGen, Vancouver, British Columbia, Canada), and monoclonal anti-Mcl-1 (BD Biosciences, San Jose, CA) primary antibodies and appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology).

Analysis of caspase activity. Activity of caspases were determined by incubating liver extracts (50 μ g) with 100 μ M site-specific tetrapeptide substrates (asp-glu-val-asp-aminomethyl-coumarin [AMC] for caspase-3, ilc-glu-threonine-asp-AMC for caspase-8 and lea-glu-his-asp-aminotrifluoromethyl-coumarin (AFC) for caspase-9 [Enzyme Systems Products, Livermore, CA]) at 37°C for 60 min. Fluorescence generated by the release of the fluorogenic group AFC or AMC upon cleavage of tetrapeptide by caspases was detected by a fluorescence spectrometer (Max Fluorescence Microplate Reader, Molecular Devices, San Diego, CA) at 355-nm excitation and 460-nm emission wavelengths. The signal intensities representative of caspase activities (unit/mg/min) were corrected for background and standardized relative to controls.

Real-time PCR analysis of gene expression. Total RNA was prepared from frozen livers by RNeasy midikit (Qiagen, Chatsworth, CA). One microgram of total RNA was treated with DNase I (Amersham Pharmacia Biotech, Inc, Newark, NJ) at 37°C for 5 min. cDNA was synthesized using random hexamers and TaqMan reverse transcription reagents (PE Applied Biosystems, Foster City, CA) according to the manufacturer's suggested protocol. Real-time PCR (TaqMan) was performed on a Perkin-Elmer/Applied Biosystems 7700 Prism using SYBR Green according to the manufacturer's instructions for quantification of relative gene expression. The following mouse-specific primers generated using Primer Express software (Applied Biosystems, Foster City, CA) were used: GAPDH, 5'-ATGCATCTGCACCACCAACT-3' (forward) and 5'-GCCCTTCCACAATGCCAAA-3' (reverse); Caspase-9, 5'-ACAAAC-TTGAGCACCAGATTCCG-3' (forward) and 5'-GCCGTGACCATTTCT-TGGC-3' (reverse); and Mcl-1, 5'-AGCCTGACTTCCCAGCTCACAA-3' (forward) and 5'-TCTCCAAGTCTTCATGGCCCTG-3' (reverse). Production of a single PCR product was confirmed using agarose gel electrophoresis, and primer efficiency was determined according to manufacturer's recommended protocol (Applied Biosystems).

Statistical analysis of data. Statistical test of significance was by ANOVA *post hoc* testing performed using the Tukey-Kramer test with a p value of ≤ 0.05 (JMP, SAS Institute, Research Triangle Park, NC).

RESULTS

Altered Regulation by PPAR α of Genes That Play Roles in Apoptosis

Wild-type and PPAR α -null mice were given three daily doses of the PP WY or gavage control (Anderson *et al.*, 2004a). Using transcript profiling, genes with known or suspected roles

in apoptosis were divided into proapoptotic and antiapoptotic genes. The genes were clustered and visualized using CLUSTER and TreeView programs (Eisen *et al.*, 1998) and are shown in Figure 1. Positive control genes *Cyp4a10* and *Acyl-Coenzyme A oxidase 1, palmitoyl* were upregulated by WY in wild-type but not PPAR α -null mice as expected (Fig. 1A, lane 1 vs. lane 3) and were expressed at lower levels in PPAR α -null mice, indicating that PPAR α is necessary for their basal level expression. The *Ppar α* gene itself was downregulated in control PPAR α -null mice, as expected. After WY exposure, proapoptotic genes (four out of nine) were upregulated and most of the antiapoptotic genes (four out of five) downregulated in wild-type but not in PPAR α -null mice (Fig. 1B, lane 1 vs. lane 3). There were no consistent changes in basal expression of these genes in control PPAR α -null mice compared to control wild-type mice (Fig. 1B, lane 2). In the proapoptotic category, there were four genes with increased abundance and two with decreased abundance. In the antiapoptotic category, there were two genes with increased abundance and one with decreased abundance. Based on this preliminary gene survey, we hypothesized that wild-type mice treated with WY would be more sensitive to the effects of apoptosis inducers than PPAR α -null mice.

Wild-Type and PPAR α -Null Mice Exhibit Differences in Sensitivity to the Apoptosis Inducer, Jo2

We first determined if there were differences in survival time between wild-type and PPAR α -null mice after a lethal dose of

Jo2. Jo2 is an antibody which recognizes and activates the Fas (Apo-1/CD95) death pathway and has been used extensively as a tool to understand mechanisms of apoptosis and fulminant hepatitis (e.g., Zhang *et al.*, 2000). Control wild-type or PPAR α -null mice had an average survival time of ~ 6 or ~ 26 h, respectively (Fig. 2A). Wild-type mice fed the WY diet exhibited decreased survival time (~ 2.5 h) compared to control wild-type mice. PPAR α -null mice fed the WY diet did not exhibit any significant differences in survival time compared to control PPAR α -null mice.

In a separate study, pathological evaluation of the livers of animals sacrificed 2 h after injection revealed that wild-type mice fed the WY diet exhibited massive hepatic hemorrhagic necrosis (Fig. 2B). The livers of wild-type mice fed the control diet exhibited mild to moderate hemorrhagic necrosis. In contrast, there were no consistent pathological changes in the livers of PPAR α -null mice fed either the control diet or the WY diet at this time point. The number of apoptotic bodies as assessed by the TUNEL assay was significantly increased 2 h following Jo2 administration in wild-type mice fed the WY diet compared to the other three groups (Figs. 2C and 2D). These results demonstrate that WY enhances sensitivity of wild-type mice to Jo2 and that PPAR α -null mice are resistant to the effects of Jo2.

Exposure to WY Activates Caspase-9 and Enhances the Effect of Jo2 on Caspase-9 and Caspase-3 Activation

Differences in the level of activated caspase-3 (12 kDa protein) were observed between wild-type and PPAR α -null

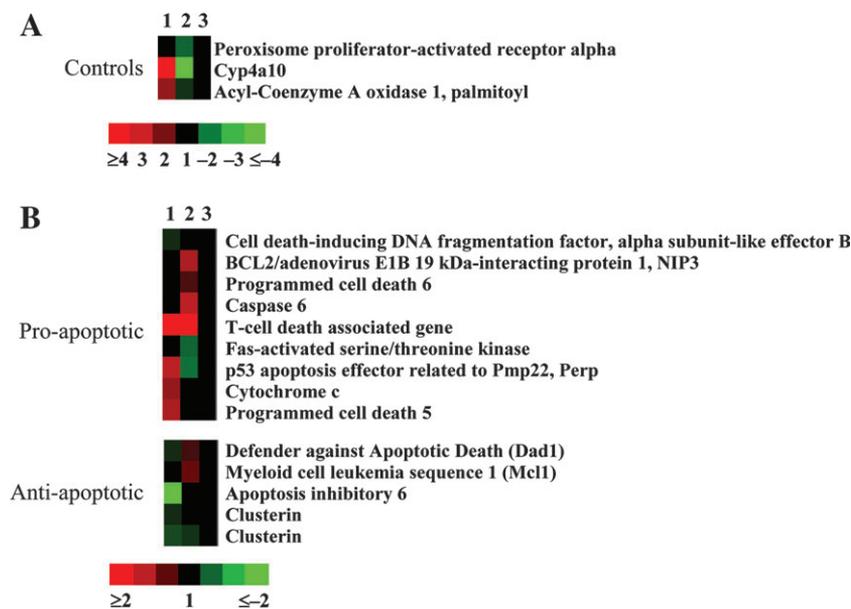


FIG. 1. Altered regulation of apoptotic genes by WY. Wild-type and PPAR α -null mice were given WY or methylcellulose each day for 3 days. On the fourth day the mice were sacrificed, and gene expression was evaluated as described in the "Materials and Methods" section. (A) Genes known to be altered by WY treatment (*Cyp4a10* and *Acyl-Coenzyme A oxidase 1, palmitoyl*) were included as positive controls with *Ppar α* . (B) Genes with known or suspected roles in apoptosis that were significantly different between two or more groups ($p \leq 0.001$) were separated into pro- and antiapoptotic genes. The two groups were clustered by hierarchical clustering using CLUSTER and visualized using TreeView. Lane 1, differences between WY-treated and control wild-type mice. Lane 2, differences between control PPAR α -null mice and control wild-type mice. Lane 3, differences between WY-treated and control PPAR α -null mice. The scales represent fold differences.

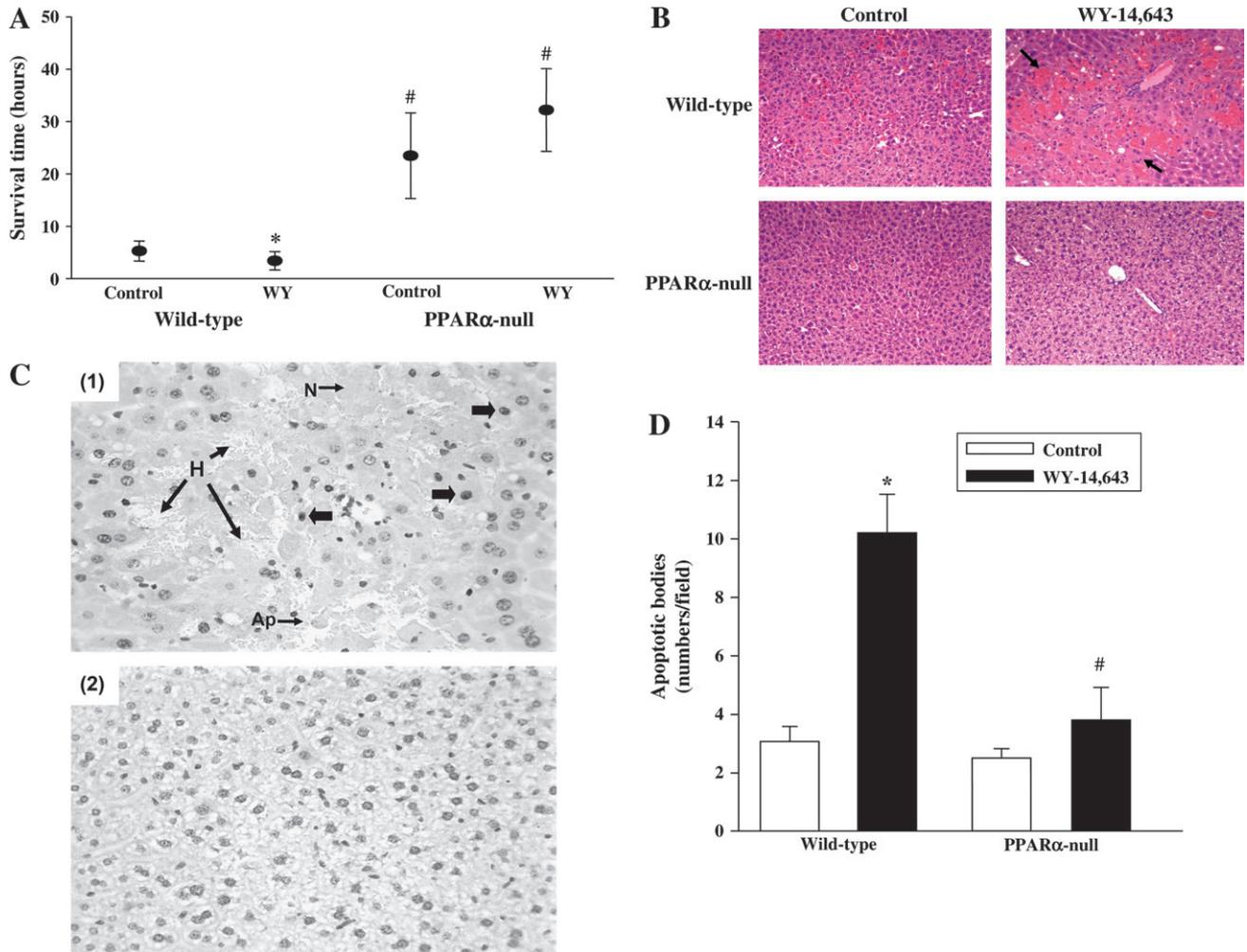


FIG. 2. PPAR α activation enhances the effect of anti-Fas antibody (Jo2) on mortality and hepatic injury. Wild-type and PPAR α -null mice were fed a control diet or a diet containing WY (0.05%) for 1 week. All mice were given one dose of the Jo2 antibody (6 μ g/kg). (A) Survival time of mice after injection of Jo2. (B) Hematoxylin and eosin staining of livers from control; WY-fed, wild-type; or PPAR α -null mice 2 h after challenge with Jo2. Arrows indicate regions of necrosis. (C) Visualization of apoptotic bodies by TUNEL staining 2 h after Jo2 injection. Photomicrographs (TUNEL staining with hematoxylin counterstain) of livers from mice killed 2 h after injection with Jo2 (6 μ g/kg): (1) extensive areas of hemorrhagic necrosis (H), scattered TUNEL-positive cells (block arrows), and necrotic hepatocytes (N) were found in wild-type, WY-fed mice. Small round apoptotic hepatocytes (Ap) were rarely seen. Mild to moderate hemorrhagic necrosis was found in the control wild-type group (not shown). (2) No significant pathology was found in either the PPAR α -null mice fed the WY diet (shown) or the control PPAR α -null mice (not shown). (D) Quantitation of apoptotic bodies. Apoptotic bodies were detected by the TUNEL assay 2 h after Jo2 injection. Data are expressed as mean \pm SD of three mice in triplicate. *Significantly different from the control group ($p < 0.05$). #Significantly different from the corresponding wild-type group ($p < 0.05$).

mice as early as 3 h after injection of Jo2. At this time robust activation was detected in wild-type mice, whereas PPAR α -null mice exhibited no consistent activation of caspase-3 (Figs. 3A and 3B). Minor increases in the activated form of caspase-3 were observed in PPAR α -null mice at 6 h, but the level of activation was a fraction of that seen in wild-type mice. Consistent with the Western blot data, enzymatic analysis of caspase-3 activity revealed activation as early as 3 h in wild-type mice (Fig. 3C). In PPAR α -null mice activation did not become significant until 6 h after exposure, and activity always remained significantly lower in PPAR α -null mice compared to wild-type mice at 3 and 6 h. Additional experiments demonstrated that caspase-8, another effector caspase of Jo2 was not

significantly altered in expression or activity after Jo2 treatment (data not shown).

Caspase-9 revealed an unusual pattern of activation. In the absence of Jo2 treatment, the 12-kDa-activated form was observed by Western blotting after WY treatment in wild-type but not PPAR α -null mice (Figs. 4A and 4B). Consistent with this, hepatocyte extracts exhibited a modest increase in caspase-9-specific activity in wild-type but not PPAR α -null mice after WY treatment (Fig. 4C). Parallel experiments did not show any changes in caspase-3 activation by Western blot analysis in the WY-treated mice in the absence of Jo2 exposure (data not shown). Challenge with Jo2 for 1.5 h augmented both the level of activated protein (Figs. 4A and 4B) and enzymatic

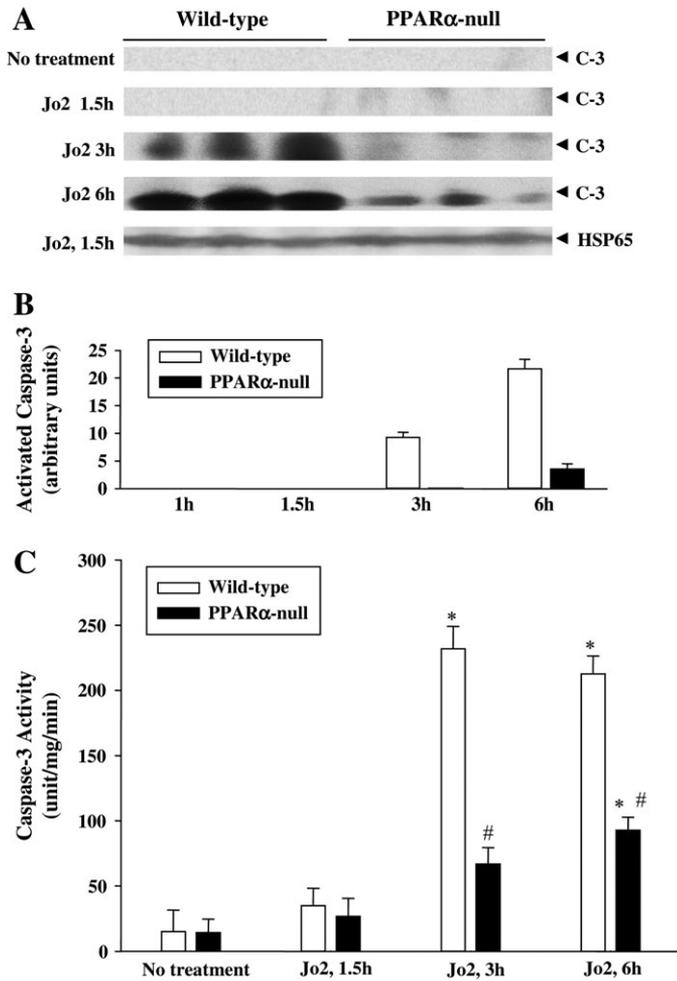


FIG. 3. PPAR α -null mice exhibit resistance to caspase-3 activation induced by Jo2. (A) Analysis of caspase-3 (C-3) activation in liver tissues assessed by immunoblot at different times after injection of Jo2. HSP65 expression was used to assess loading of the lanes and is shown for the 1.5-h time point. Other time points gave similar loading profiles (data not shown). (B) Blots were quantitated as described in the "Materials and Methods" section. (C) Caspase-3 activity in liver tissues after injection of Jo2. Caspase-3 activity was measured by the release of the AMC fluorochrome from peptide substrate (DEVD).

activity (Fig. 4C) of caspase-9. The levels of procaspase-9 were markedly decreased under these conditions. The other groups of mice did not exhibit significant increases in caspase-9 activation at this time point. These results demonstrate that (1) the level of caspase-3 activation parallels the sensitivity differences in apoptosis induction in wild-type and PPAR α -null mice and (2) exposure to a PPAR α agonist in the absence of Jo2 exposure results in partial cleavage of the caspase-9 proenzyme and increases in caspase-9 activity.

Exposure to WY Decreases the Expression of the Caspase-9 Effector Protein, Mcl-1

A number of pro- and antiapoptotic proteins in the Bcl-2 family regulate cytochrome c release from mitochondria. The

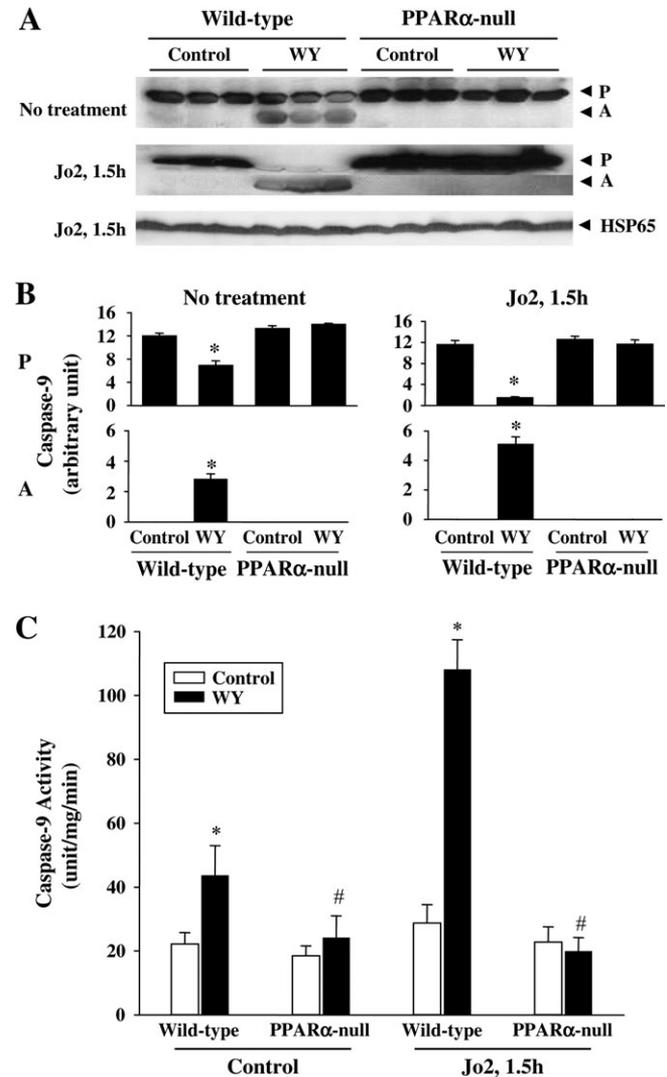


FIG. 4. Exposure to the PPAR α agonist WY results in activation of caspase-9. (A) Analysis of caspase-9 activation in liver tissues assessed by immunoblot at different times after injection of Jo2. Pro- (P) and activated (A) forms of caspase-9 are indicated. HSP65 expression was used to assess loading of the lanes. (B) Blots were quantitated as described in the "Materials and Methods" section. (C) Caspase-9 activity in liver tissues before and after injection of Jo2. Caspase-9 activity was measured by the release of the AFC fluorochrome from peptide substrate (lea-glu-his-asp).

transcript profiling experiments described above indicated that Bcl-2 family members, Bax, Bad, Bak1, Bag3, Bnip2, Dap3, and Bim as well as Bcl-2 itself, were unaffected by WY exposure in wild-type or PPAR α -null mouse livers (data not shown). We also examined the protein levels of some of the Bcl-2 family members by Western blots and showed that under acute exposure conditions (three daily doses of WY), Bcl-xL, Bcl-2, Bax, and Bid protein levels do not change after WY exposure (data not shown). However, Mcl-1 mRNA expression was significantly decreased by WY in wild-type mice (Fig. 5A). PPAR α -null mice fed either the WY-containing or control

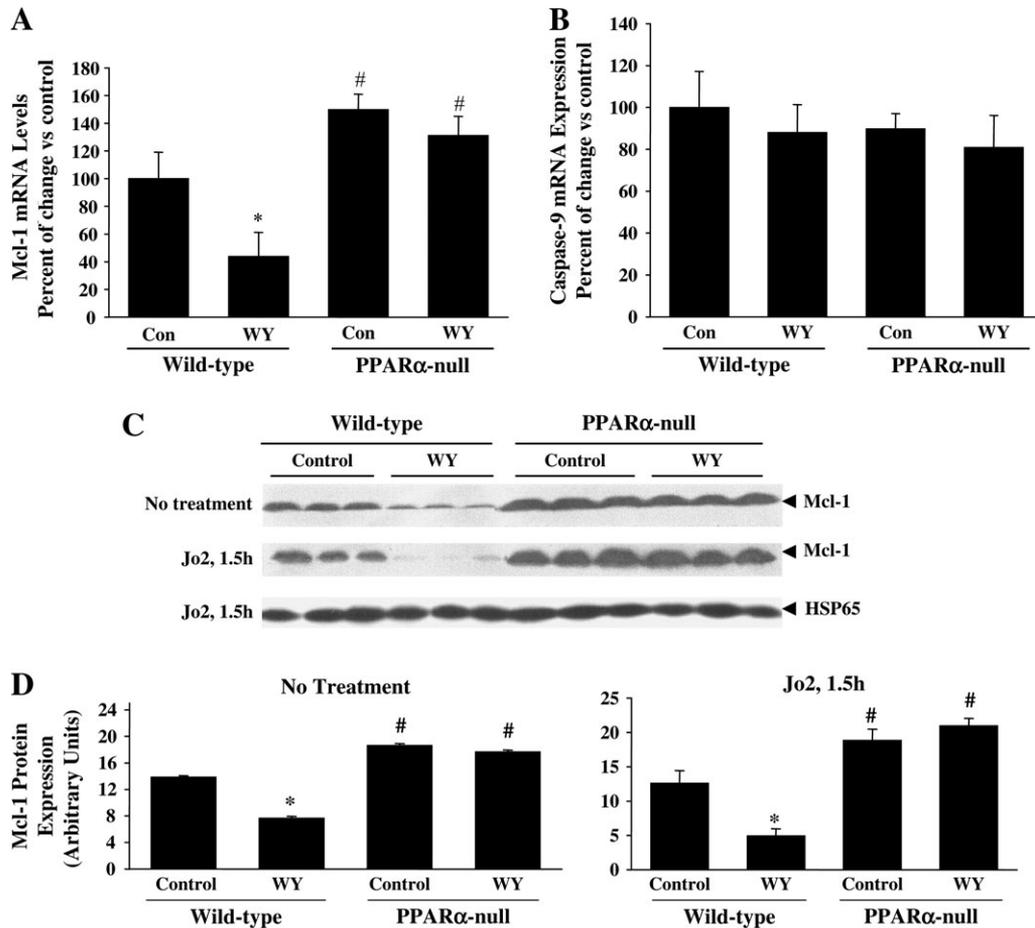


FIG. 5. PPAR α decreases the expression of the caspase-9 effector Mcl-1. (A) Analysis of Mcl-1 mRNA expression by real-time RT-PCR (TaqMan). Data were mean \pm SD of three mice in triplicate. (B) Analysis of caspase-9 mRNA expression by TaqMan. (C) Analysis of Mcl-1 protein expression by Western blot. (D) Quantitation of Mcl-1 protein before (left) and after (right) treatment with Jo2 for 1.5 h. HSP65 expression was used to assess loading of the lanes. Blots were quantitated as described in the "Materials and Methods" section.

diets exhibited higher levels of Mcl-1 than their wild-type counterparts. WY exposure did not affect the mRNA expression of caspase-9 in wild-type or PPAR α -null mice (Fig. 5B), indicating that increases in the activated form of caspase-9 upon WY exposure were unlikely due to changes in mRNA abundance. Altered expression of the Mcl-1 protein paralleled that of the Mcl-1 mRNA (Figs. 5C and 5D). Expression was decreased after WY exposure in wild-type mice; PPAR α -null mice exhibited increased expression of the Mcl-1 protein independent of exposure. Exposure of mice to Jo2 did not appreciably change the expression of Mcl-1 compared to mice not treated with Jo2 (Figs. 5C and 5D).

We next determined if the activation of caspase-9 and downregulation of Mcl-1 after WY exposure was compound specific by examining the responses after exposure to another PP. TCA, a metabolite of the industrial solvent trichloroethylene was given to wild-type and PPAR α -null mice in the drinking water (2 g/l) for 1 week. Under these conditions, typical markers of PP exposure were elevated (Laughter *et al.*, 2004).

Expression of procaspase-9 in the livers of wild-type mice was decreased in parallel with increased expression of activated caspase-9 (Figs. 6A and 6B). There were no changes in the expression of the pro- or activated forms of caspase-9 in PPAR α -null mice. Mcl-1 protein expression was decreased in wild-type but not PPAR α -null, TCA-treated mice (Figs. 6A and 6B).

As RXR agonists induce many of the same responses in mouse livers as PPs (Anderson *et al.*, 2004a), we determined if the RXR-specific agonist AGN-194,204 also behaved similarly to a PPAR α agonist. Exposure to the AGN compound for 3 days did not alter the expression of caspase-9 or Mcl-1 (data not shown). These results indicate that decreases in Mcl-1 expression and increases in caspase-9 activation are responses specific to PP exposure, mediated by PPAR α .

PPAR α -Null Mice Are Resistant to Another Inducer of Apoptosis

We determined if differences in sensitivity between wild-type and PPAR α -null mice were specific to Jo2 or if another

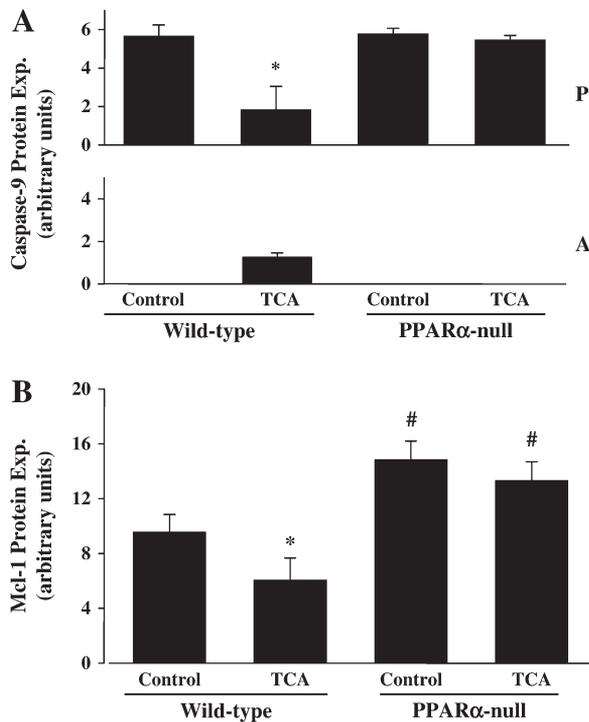


FIG. 6. The PPAR α agonist TCA activates caspase-9 and downregulates Mcl-1 protein expression. (A) Detection of activated caspase-9. Both the pro (P) and activated (A) forms of caspase-9 were detected by Western blots after treatment with TCA. Blots were quantitated as described in the "Materials and Methods" section. (B) Analysis of Mcl-1 protein expression after treatment with TCA determined by Western blots.

inducer of apoptosis would exhibit the same behavior. We treated mice with Con A which induces T-cell activation and production of cytokines including TNF- α (Batey and Wang, 2002). Only wild-type mice pretreated with WY exhibited an

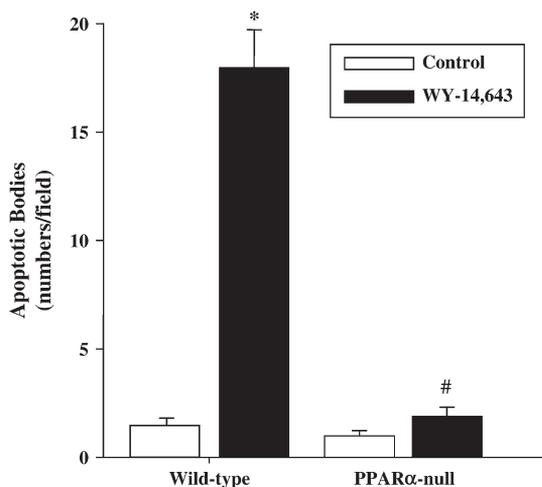


FIG. 7. PPAR α -null mice exhibit resistance to apoptosis induction by Con A. Wild-type and PPAR α -null mice with and without pretreatment with WY were injected with 25 mg/kg Con A. Apoptotic foci were counted in liver tissues by pathological examination.

increased number of apoptotic bodies after Con A exposure (Fig. 7). These results indicate that PPAR α -null mice are resistant to hepatocyte apoptosis induced by Con A.

DISCUSSION

PPAR α is a major regulator of lipid metabolism in tissues that require fatty acid oxidation and mediates the hepatocarcinogenic effects of PP through alterations in hepatocyte growth (Corton *et al.*, 2000; Klaunig *et al.*, 2003). To further define the mechanism of PP-induced effects, we used transcript profiling to catalog gene expression differences between wild-type and PPAR α -null mice treated with the potent PP, WY. Genes that play functional roles in apoptosis were altered after treatment in wild-type but not PPAR α -null mice. Based on this pattern, we hypothesized that PPAR α activation would enhance susceptibility to apoptosis inducers. This challenged the assumption that PPs generally suppress apoptosis in the liver (Gill *et al.*, 1998; Hasmall *et al.*, 2000; James *et al.*, 1998).

Wild-type and PPAR α -null mice were treated with two inducers of hepatocyte apoptosis whose mechanisms likely converge at alterations in the mitochondrial permeability transition, release of cytochrome c, assembly of the apoptosome, activation of the regulatory caspase-9, and activation of downstream effector caspases. We showed that when compared to wild-type control mice, WY increased sensitivity of wild-type mouse hepatocytes *in vivo* to the Fas-activating Jo2 antibody or the T-cell activator Con A. Increased sensitivity depended on a functional PPAR α . Mice nullizygous for PPAR α were resistant to the apoptosis-inducing effects of Jo2 and Con A. Differences in sensitivity between wild-type and PPAR α -null mice to Jo2-induced apoptosis correlated with differences in the levels of activated caspase-9 and caspase-3. These studies provide evidence that PPAR α activation positively regulates apoptosis in the liver. Our results are consistent with the increases in hepatocyte apoptosis observed 1 and 13 weeks after WY exposure in mice (Burkhardt *et al.*, 2002). Together, the data suggest that suppression of apoptosis as observed earlier after an acute PP exposure (James *et al.*, 1998) is not a general feature of PP exposure and thus may not be mechanistically linked to PP-induced liver cancer.

A model of PPAR α -dependent regulation of hepatocyte apoptosis by PPs is shown in Figure 8. Central to this model is our finding that the expression of the antiapoptotic gene Mcl-1 is downregulated in the liver after exposure to PPs. Forced overexpression of Mcl-1 in cell lines led to suppression of apoptosis stimulated by etoposide, UV irradiation, growth factor withdrawal, and actinomycin D (Reynolds *et al.*, 1994; Zhang *et al.*, 2002; Zhou *et al.*, 1997), whereas Mcl-1 inhibition, through expression of antisense RNA led to increases in spontaneous and hypoxia-induced apoptosis (Derenne *et al.*, 2002; Leuenroth *et al.*, 2000; Moulding *et al.*, 2000). We showed that upon exposure to WY or another

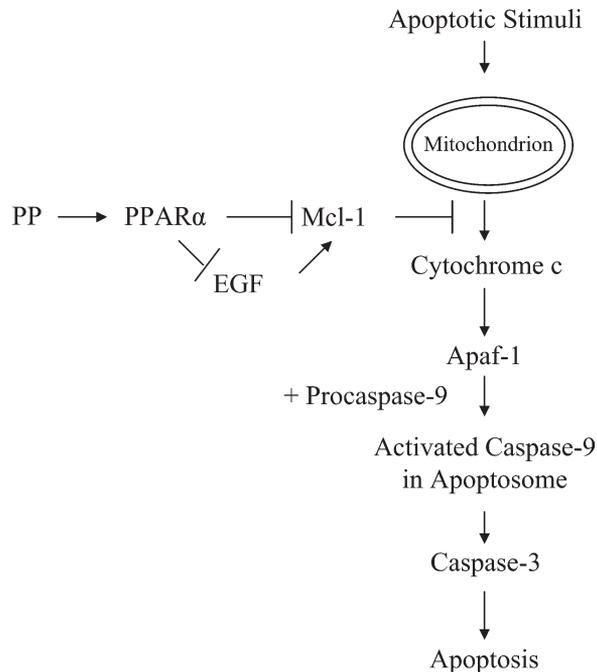


FIG. 8. Model of PP effects on hepatocyte apoptosis. See the text for details.

PP, TCA resulted in downregulation of mRNA and protein levels of Mcl-1. This downregulation required PPAR α as PPAR α -null mice exposed to WY or TCA did not exhibit differences in expression compared to control PPAR α -null mice. In addition, Mcl-1 expression was increased in control PPAR α -null mice compared to wild-type mice. This type of regulation indicates that PPAR α controls constitutive as well as PP-regulated expression of Mcl-1 in the liver similar to that observed for negatively regulated acute-phase protein genes (Corton *et al.*, 1998). Previously published data indicate that the Mcl-1 protein is coregulated with other Bcl-2 family members after PP exposure. Bcl-2 and Bag1 were elevated at 3 days but not 1 day after WY exposure in the mouse liver, and Bcl-2 was elevated and Bak was downregulated in mouse primary hepatocytes after exposure to the PP nafenopin (Christensen *et al.*, 1999). In human primary hepatocytes there is evidence for opposite regulation of Bcl-2 as the mRNA expression was reduced by the PP fenofibrate (Kubota *et al.*, 2005).

There are at least two possible mechanisms as to how Mcl-1 might be regulated by PPAR α . PPAR α could regulate Mcl-1 expression indirectly by binding and sequestration of a factor essential for Mcl-1 expression, although there is no historical mechanistic information to support this. Alternatively, PPAR α could negatively regulate growth factor pathway signaling. Mcl-1 is positively regulated by growth factors including interleukins 3, 5, 6 and epidermal growth factor (EGF) that activate JAK/STAT- and phosphatidylinositol-3 kinase/Akt-dependent pathways (Leu *et al.*, 2000; Wang *et al.*, 1999)

presumably to suppress apoptosis during times when cell proliferation is active. Serum withdrawal results in suppression of Mcl-1 expression and increased apoptosis (Klampfer *et al.*, 1999; Zhou *et al.*, 1997). Our transcript profile experiments showed that EGF receptor expression is strongly downregulated after a 1-week exposure to WY (Anderson *et al.*, 2004b) consistent with decreases in EGF receptor binding capacity and expression in livers from PP-exposed mice and rats (Gupta *et al.*, 1988). The evidence supports a model in which suppression of Mcl-1 expression by PP requires PPAR α -dependent suppression of EGF signaling through downregulation of EGF receptor expression.

We unexpectedly found that exposure to WY or TCA results in caspase-9 cleavage and increases in basal activity of caspase-9 in liver extracts in the absence of apoptosis induction. Caspase-9 activation and cleavage were not observed in PP-treated PPAR α -null mice. Upon exposure to Jo2, the remaining full-length procaspase-9 was almost completely lost with a parallel increase in the cleaved form in WY-treated, wild-type mice. However, only after Jo2 exposure did we observe increases in caspase-3 cleavage and activation. These results imply that a subset of caspase-9 proteins are cleaved after PP exposure, and the cleaved caspase-9 is unable to activate caspase-3 until stimulation by apoptosis inducers. To our knowledge, this is the first example of chemical-induced caspase-9 cleavage in the absence of overt apoptosis and may explain the increased sensitivity of WY-treated, wild-type mice to apoptosis induction. Although the exact mechanism of caspase-9 cleavage is not known, a number of studies have shown a correlation between Mcl-1 downregulation and increases in caspase-9 activation (Boucher *et al.*, 2000; Guzey *et al.*, 2002; Tong *et al.*, 2002) consistent with our model.

In stark contrast to the ability of PPs to induce liver cancer, PPs have the ability to suppress the growth of different types of animal and human cancers. Early indications that PP can suppress growth of preneoplastic lesions came from studies in which rats were treated with the DNA-damaging agent diethylnitrosamine, an inducer of different types of preneoplastic foci in the liver. Subsequent treatment of these rats with a PP resulted in suppression of both γ -glutamyl transpeptidase-positive and ATPase-deficient foci (Cattley and Popp, 1989; DeAngelo *et al.*, 1985). More recently, treatment of mice with two structurally distinct PP reduced the number and size of skin papillomas (Thuillier *et al.*, 2000). A wide variety of animal and human tumor cell lines that express PPARs are sensitive to PPAR agonist exposure *in vitro* (Corton *et al.*, 2000). Growth inhibition of these cell lines occurred through distinct mechanisms, including increases in necrosis (Kubota *et al.*, 1998), apoptosis (Canuto *et al.*, 1998; Elstner *et al.*, 1998), and growth arrest (Ferrandina *et al.*, 1997). Our findings in the mouse liver identify a potential mechanism for therapeutic interventions by which PP exposure through PPAR α can increase sensitivity to apoptosis induction.

ACKNOWLEDGMENTS

We thank the Animal Care Unit and Necropsy and Histology Staff for assistance with animal care and necropsies, Dr Frank Gonzalez for wild-type and PPAR α -null mice to build our mouse colony, Mr Donald Joyner for assistance with photography, Dr Rosh Chandraratna for the AGN compound, and Drs Kevin Gaido and Elena Kleymanova for reviewing the manuscript.

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