Role of peroxisome proliferator-activated receptor α in altered cell cycle regulation in mouse liver

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The mechanisms underlying peroxisome proliferator-induced hepatocarcinogenesis are unclear but are mediated by the peroxisome proliferator-activated receptor α (PPARα). To determine the role of PPARα in the mechanisms of hepatocarcinogenesis, the effect of Wy-14,643 on expression patterns of acyl CoA oxidase (ACO) and proteins involved in cell proliferation in the PPARα-null mouse were evaluated. ACO, CDK-1, CDK-2, CDK-4, PCNA and c-myc proteins were significantly increased in wild-type mice fed Wy-14,643 for 5 weeks or 11 months, as compared with controls. This effect was not observed in Wy-14,643-treated PPARα-null mice. Expression patterns of cyclin B1, cyclin D, cyclin E and p53 were not different in any of the groups. mRNAs encoding CDK-1, CDK-4, cyclin D1 and c-myc were also increased in wild-type mice fed Wy-14,643 but not in PPARα-null mice. These results indicate that the increase in CDK-1, CDK-4 and c-myc may be caused by an increase in transcription that is mediated directly or indirectly by PPARα. Thus PPARα-dependent alterations in cell cycle regulatory proteins induced by peroxisome proliferators are likely to contribute to the hepatocarcinogenicity of peroxisome proliferators.

Introduction

Peroxisome proliferators are a diverse class of chemicals that induce a pleiotropic response and hepatocarcinogenesis when administered to rodents (1–3). Short-term administration of peroxisome proliferators results in hepatomegaly and altered expression of genes encoding peroxisomal, mitochondrial and microsomal lipid metabolizing enzymes (reviewed in refs 4, 5). Among the best characterized of these proteins is the peroxisomal β-oxidizing enzyme acyl CoA oxidase (ACO), the rate limiting enzyme responsible for initiating peroxisomal fatty acyl CoA oxidation (6, 7). In contrast, expression of genes encoding apolipoprotein A-I, C-III and others are reduced after exposure to peroxisome proliferators (8–10).

Peroxisome proliferator-induced changes in gene expression are mediated by the peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear receptor superfamily. On activation by peroxisome proliferators, PPARα forms a heterodimer with retinoid-X-receptor and the resulting complex then binds to peroxisome proliferator responsive elements (PPRE) located in promoter regions of target genes that are regulated by PPARα. There is a growing list of genes that contain PPREs whose expression is altered by PPARα (reviewed in ref. 11).

Long-term administration of peroxisome proliferators causes hepatocarcinogenesis in rodents. The mechanisms underlying this effect are unclear but could be the result of alterations in gene expression mediated by PPARα. Most responses induced by peroxisome proliferators in rodent liver are dependent on PPARα. For example, hepatomegaly, increases in cell proliferation as measured by increases in replicative DNA synthesis, altered expression of genes encoding peroxisomal, mitochondrial and microsomal enzymes, and hepatocarcinogenesis are all responses induced by peroxisome proliferators that require PPARα (12–14). While it is clear that PPARα mediates these effects, the precise mechanisms are unknown.

Two non-exclusive hypotheses have been postulated to account for peroxisome proliferator-induced carcinogenicity. Peroxisome proliferators cause large increases in hepatic ACO expression that is not accompanied by a similar increase in catalase. Thus increased intracellular H2O2 resulting from ACO catalytic activity has been hypothesized to contribute to the carcinogenic effect of peroxisome proliferators (15) since H2O2 is capable of causing DNA damage through the formation of adducts such as 8-hydroxydeoxyguanine or strand breaks. Sustained alterations in cell proliferation (16, 17), apoptosis (18, 19), or both, also occur as a result of peroxisome proliferator administration, and these changes have been postulated to contribute to the underlying mechanism of peroxisome proliferator-induced hepatocarcinogenicity (20). In support of the latter hypothesis, Wy-14,643 was recently shown to cause a discordant expression of a number of cell cycle control proteins, including proliferating cell nuclear antigen (PCNA), cyclins and cyclin-dependent kinases (CDK) (21–23). In this work, the effect of Wy-14,643 on cell cycle regulatory proteins and ACO expression patterns in wild-type and PPARα-null mice was compared to determine whether these effects are dependent on PPARα under conditions known to result in liver carcinogenesis in wild-type mice but not in PPARα-null mice.

Materials and methods

Dietary treatment and tissue collection

Tissue samples used in this study were obtained from mice treated as described below from a previously published experiment (14). Seven- to eight-week-old, wild-type (+/+) or PPARα-null (−/−) male mice on an Sv/129 background (F3 generation) were housed 4–5 animals per cage in a temperature- and light-controlled environment (T = 25°C, 12 h light–dark cycle). Mice were fed a pelletized mouse chow containing either 0.0 (control) or 0.1% 4-chloro-6-(2,3-xylidino)-pyrimidinylthioacetic acid (Wy-14,643) provided ad libitum for either 5 weeks or 11 months. After treatment, the mice were euthanized and necropsied. Liver samples were obtained and snap-frozen until further analysis.

Protein analysis

Nuclear extracts were prepared from livers of mice fed for either 5 weeks or 11 months as previously described (24). Hepatic nuclear extracts from (+/+)...
mice fed the Wy-14,643 diet for 11 months were prepared from tissue with
grossly visible tumors. From 10 to 50 µg of protein were separated by
SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane
electroblotting in standard Tris–glycine buffer. For analysis of pure ACO
µ
no grossly visible tumors. From 10 to 50
mice fed the Wy-14,643 diet for 11 months were prepared from tissue with
proteins of ~70 kDa, 10
described above. Proteins were visualized with Coomassie Blue stain using
of cyclin B1, PCNA, c-
myc
and ACO. Immunodetection was performed
proteins of ~70 kDa, 10 µg of protein was loaded for western blot analysis
of cyclin B1, PCNA, c-myc and ACO. Immunodetection was performed
by using specific primary antibodies followed by conjugation with a secondary,
horseradish peroxidase-labeled antibody and then detected with a chemilumin-
escence kit (ECL; Amersham Life Science, Cleveland, OH). The primary
antibodies used included those raised against mouse ACO, cyclin B1, cyclin
D1, cyclin E, CDK-1 (cdc2 p34), CDK-2, CDK-4, PCNA, p53 and c-myc.
Antibodies were purchased commercially (Santa Cruz Biotechnology, Santa
Cruz, CA) except for the ACO antibody, which was developed and character-
ized in an earlier report (25). Analysis of liver samples from four mice per
group was performed, although representative immunoblots from two separate
mice from each group are presented in the figures shown in the text.

mRNA analysis
RNA samples from liver with no grossly visible tumors were obtained and
analyzed essentially as previously described (13). Seven cDNA probes
previously described were used for sequential northern blot analysis, including
mouse CDK-1, CDK-2, CDK-4, cyclin B1, cyclin D1, c-myc and β-actin
(13,26–30). A mouse PCNA cDNA was obtained by reverse transcription
PCR from 0.5 µg of total RNA isolated from Wy-14,643-treated mouse liver.
The PCR primers selected were based on the published cDNA sequence of
mouse PCNA (31). The second strand cDNA was amplified by subsequent
PCR with the designed primers. The forward PCR primer for mouse PCNA
was 5’-CTCCAGGTCCTCTCTTTCGTTACGCATTTGTCG-3’ (271–295). The reverse
PCR primer for mouse PCNA was 5’-CTCCATCATCTCTCAATGTGGAGGAG-
3’ (921–988). The amplified cDNA fragment for mouse PCNA was 651 bp
corresponding to nt 271–921 and its identity was confirmed by sequencing.

Results
Analysis of total hepatic nuclear proteins separated by SDS–PAGE revealed significant differences in expression patterns in (+/+ ) mice fed Wy-14,643 for either 5 weeks (Figure 1A) or 11 months (Figure 1B) compared with untreated controls. A protein with an apparent mol. wt of ~70 kDa was very abundant in liver samples from (+/+) mice fed Wy-14,643 compared with control samples, and the increase in staining intensity was highest among all other proteins visualized on the gel (Figure 1). Nuclear proteins from (+/+) mice fed Wy-14,643 also had higher levels of at least four other proteins with apparent mol. wts of ~65, ~50, ~43 and ~35 kDa compared with untreated control samples (Figure 1). In addition, several

![Fig. 1.](image)

Fig. 1. Nuclear proteins from hepatic samples (20 µg/lane) were separated by SDS–PAGE and stained with Coomassie Blue. Molecular weight markers are indicated with arrows. Representative liver samples were used from two mice of each genotype, (+/+) or (−/−), fed either a control diet or one containing 0.1% Wy-14,643 for 5 weeks (A) or 11 months (B).

![Fig. 2.](image)

Fig. 2. Western analysis of ACO in hepatic samples (10 µg/lane) were separated by SDS–PAGE and transferred to nitrocellulose membranes for detection with an anti-ACO polyclonal antibody. Representative liver samples from two mice of each genotype, (+/+) or (−/−), fed either a control diet or one containing 0.1% Wy-14,643 for the indicated time period were used. Apparent mol. wts of upper and lower bands are ~70 and ~50 kDa, respectively.
both the ~70 kDa and ~50 kDa subunits of ACO (Figures 2 and 4). Coomassie staining of ACO showed that this sample contained much less of the ~70 kDa subunit than the ~50 kDa protein. Further, the staining intensity correlated well with the immunoreactivity for the respective subunits of ACO (Figure 4).

Northern blot analysis was consistent with the increase in CDK-1, CDK-4 and c-myc expression observed as a result of Wy-14,643 feeding. Increased levels of CDK-1, CDK-4 and c-myc mRNA were found after 5 weeks of feeding Wy-14,643 to wild-type mice, and this effect was not observed in PPARα-null mice fed with similar diets (Figure 5). In contrast to results obtained with western analysis, which revealed no change in protein, northern analysis revealed a significant increase in cyclin D1 mRNA in (+/+) or (−/−) mice fed either a control diet or one containing 0.1% Wy-14,643 for the indicated time period were used. Antibodies used are indicated on the left, and the apparent mol. wts of the indicated protein are listed on the right.

![Fig. 3. Western analysis of cell proliferation proteins.](image)

![Fig. 4. Pure ACO is immunoreactive with unrelated antibodies.](image)

![Fig. 5. Northern analysis of hepatic mRNA.](image)

### Discussion

In wild-type mice fed Wy-14,643, standard Coomassie staining of hepatic nuclear proteins revealed a large increase in two proteins with apparent mol. wts of ~50 and ~70 kDa that were both immunoreactive with a polyclonal anti-ACO antibody. While ACO is predominantly a peroxisomal or cytosolic protein, this observation is consistent with previous studies demonstrating that peroxisome proliferators can induce ACO to levels ~20-fold higher than controls (6,33). The nuclear preps used in this work probably contained contaminating ACO because of the significant induction by Wy-14,643, as confirmed by detection with an ACO antibody. Higher levels of ACO were found in liver samples from wild-type mice fed Wy-14,643 for either 5 weeks or 11 months, before and after hepatocellular carcinomas were present, respectively (14). Further, this induction was not observed in the PPARα-null mice treated with Wy-14,643. This observation is consistent with previous reports showing a lack of an increase in ACO mRNA and protein levels in PPARα-null mice after treatment with peroxisome proliferators (12,13), which is because the ACO gene has a functional PPRE in its promoter (34).

Higher levels of hepatic CDK-1, CDK-2, CDK-4, PCNA and c-myc were found in wild-type mice fed Wy-14,643 for 5 weeks or 11 months as compared with untreated controls. For CDK-1, CDK-4 and c-myc, this effect may be caused by an increase in transcription since mRNAs for these proteins were elevated in response to Wy-14,643. Discordant expression of cell cycle regulatory proteins was also reported in rats treated with peroxisome proliferators (12,13), which is because the ACO gene has a functional PPRE in its promoter (34).

### Role of PPARα in altered cell cycle regulation

In wild-type mice fed Wy-14,643, standard Coomassie staining of hepatic nuclear proteins revealed a large increase in two proteins with apparent mol. wts of ~50 and ~70 kDa that were both immunoreactive with a polyclonal anti-ACO antibody. While ACO is predominantly a peroxisomal or cytosolic protein, this observation is consistent with previous studies demonstrating that peroxisome proliferators can induce ACO to levels ~20-fold higher than controls (6,33). The nuclear preps used in this work probably contained contaminating ACO because of the significant induction by Wy-14,643, as confirmed by detection with an ACO antibody. Higher levels of ACO were found in liver samples from wild-type mice fed Wy-14,643 for either 5 weeks or 11 months, before and after hepatocellular carcinomas were present, respectively (14). Further, this induction was not observed in the PPARα-null mice treated with Wy-14,643. This observation is consistent with previous reports showing a lack of an increase in ACO mRNA and protein levels in PPARα-null mice after treatment with peroxisome proliferators (12,13), which is because the ACO gene has a functional PPRE in its promoter (34).

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The mechanism by which PPARα regulates CDK-1, CDK-2, CDK-4, PCNA, cyclin D1 and c-myc is unclear from this study. It is important to point out that differences in expression
of cyclin D protein were not observed, whereas a significant difference in cyclin D1 mRNA was noted in Wy-14,643-treated (+/++) mice that was not seen in (−/−) mice. This disparity may be because of the inability of the commercial antibody to differentiate among the three isoforms D1, D2 and D3 whereas the cDNA probe was specific for the D1 form of this mRNA. Further, since no differences in the mRNAs for CDK-2 and PCNA were detected, the increased levels of these proteins in response to Wy-14,643 may be the result of post-transcriptional mechanisms. Indeed, PCNA expression has been reported to be affected by post-transcriptional mechanisms (37,38). In contrast, the induction of CDK-1, CDK-4 and c-myc appears to be because of increased transcription that is directly or indirectly dependent on PPARα. Whether the increase in CDK-1, CDK-4 and c-myc mRNA is the result of the presence of a PPRE in the promoter region of these genes or because of other upstream or downstream PPARα-dependent processes remains to be determined.

Although significant differences in Wy-14,643-induced changes in proteins involved in cell proliferation and cell cycle control were found, the expression pattern was distinctly different from other reports (21–23). Differences in the levels of cyclin B1, cyclin E or p53 as a result of treatment with Wy-14,643 were not observed. This disparity may be because of differences between mice (present study) and rats (previously reported). The mechanisms underlying this effect need to be determined.

More importantly, immunoreactive proteins with apparent mol. wts of both ~50 and ~70 kDa were detected in hepatic, nuclear proteins from Wy-14,643-treated wild-type mice with commercial antibodies against CDK-1, cyclin B1, c-myc and PCNA. The presence of the ~70 kDa protein has been postulated to represent a complex of cyclins, CDKs and other cofactors involved in cell cycle regulation (e.g. p21) (21,22). This hypothesis was based on results from gel filtration experiments coupled with ELISA detection of CDKs and PCNA in collected fractions. Alternatively, we hypothesized that the immunoreactive ~70 kDa protein is the result of the large induction of ACO, which could cause a concomitant increase in non-specific background of immunodetection. A large induction of ACO with apparent mol. wts of both ~50 and ~70 kDa using a specific polyclonal antibody was found, and this protein was a major band detected in total nuclear proteins on SDS-PAGE by Coomassie staining. Further, a relatively small quantity of pure ACO was found to be immunoreactive with commercially available antibodies against PCNA, CDK-1, cyclin B1 and c-myc. Thus these results suggest that the large increase in ACO observed on treatment with Wy-14,643 causes increased non-specific binding because of protein–antibody interactions that can interfere with differentiation of proteins with apparent mol. wt of ~70 kDa.

The most difficult interpretation from these data is how these sustained alterations in cell cycle control proteins and others involved in cell proliferation contribute to the carcinogenic properties of peroxisome proliferators. Cyclin-dependent kinases, cyclins, PCNA and c-myc have critical and complex roles in cell proliferation. For example, the kinase activity of CDKs is regulated by phosphorylation, dephosphorylation or both, and by complexing with cyclins (39,40). Transcriptional regulation of CDK expression has also been suggested (39–43). Further, substrates for phosphorylation by CDKs are variable and dependent on the phase of the cell cycle (44–46). In addition, fluctuations in the cellular content of CDKs and cyclins occur throughout the cell cycle, including the major transitions between G1, S, G2 and M, and roles for many of these proteins are overlapping (43,47). Given the complexity in the roles of cell cycle control proteins and the fact that only mRNA and protein levels were measured in this study, the present data do not yield solid evidence for any specific roles for PPARα other than that it is required for the induction of these proteins. However, increased expression of CDKs, cyclins, PCNA and c-myc have all been reported in cancer cells (48–51). In fact, increased expression of PCNA and CDK-1 have been used as biomarkers of increased cell proliferation (52,53). Whether an increase in cyclin or CDK expression is a cause or an effect of carcinogenesis has not been elucidated. However, our results provide more evidence that this imbalance is present prior to the formation of tumors, which is suggestive of a causal relationship. Specific evidence to support the idea that increased expression of cyclins leads to tumor formation comes from mice where targeted overexpression of cyclin D1 leads to mammary tumors (54).

Differential expression of proteins involved in cell replication has been reported in hepatocellular carcinomas (55–57). In the current study, analysis of proteins involved in cell replication was not compared between liver tumors and the surrounding parenchyma. However, in tumor-bearing liver from rats fed Wy-14,643 for 78 weeks, expression of c-myc, c-erb and PPARα mRNA is increased as a result of Wy-14,643 administration compared with control liver, yet the difference in the level of these mRNAs between tumor and the surrounding parenchyma is not substantial (58). Thus, the mechanisms underlying the difference in cell proliferation in tumors relative to surrounding non-tumor liver (59) is unknown.

It is possible that the alterations in proteins caused by Wy-14,643 that regulate transitions in the cell cycle may cause dysregulation by improper timing of signals (i.e. G1 signals during G1 transition, and vice versa) thus resulting in preneoplastic cells dedifferentiating into tumor cells (22). Alterations that result in accelerated and sustained cell proliferation may predispose cells that would normally undergo apoptosis to differentiate into tumor cells (17,36). For example, the sustained PPARα-dependent induction of ACO that can result in increased intracellular H2O2 may cause DNA damage in cells that becomes ‘fixed’ as a mutation in the presence of increased cell proliferation. Interestingly, c-myc may have a critical role in both cell cycle progression and in apoptosis (60,61). Finally, Kupffer cell production of tumor necrosis factor (TNFα) has been implicated in the mechanism of peroxisome proliferator-induced mitogenesis (62,63). Thus a role for TNFα in altering cell cycle control proteins cannot be eliminated. Clearly, elucidating the specific role or roles of these sustained imbalances in cell cycle control proteins and other proteins involved in cell proliferation will provide invaluable insight into the mechanism of peroxisome proliferator-induced hepatocarcinogenesis.

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