Mechanisms of Resistance of Hepatocyte Retinoid X Receptor α-Null Mice to WY-14,643-induced Hepatocyte Proliferation and Cholestasis*

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Peroxisome proliferators, such as the lipid-lowering fibrates that function as agonists for peroxisome proliferator-activated receptor α (PPARα), induce liver tumors in rodents and may produce cholestasis in humans. Considerable attention has focused on peroxisome proliferator-induced hepatocellular carcinoma, a phenomenon not noted in man, whereas limited studies examine fibrates and other therapeutic drugs that induce cholestasis, a common finding in humans. Moreover, the mechanisms by which fibrates induce hepatocyte proliferation and cholestasis are still not fully understood. We have examined the role of hepatocyte retinoid X receptor α (RXRα), an essential partner of PPARα, in modulating WY-14,643-induced hepatocyte proliferation and cholestasis. WY-14,643 treatment induced hepatomegaly in wild type (WT) mice that was also accompanied by induction of the expression of cyclins D1, D3, A2, and B1 and Cdc2 as well as inhibition of Wee 1. Such changes were either absent or greatly reduced in hepatocyte RXRα-null mice. Furthermore, neither WY-14,643 treatment nor RXRα deficiency affected apoptosis, indicating the importance of PPARα/RXRα in regulating Wee 1-mediated Cdc2/cyclin B1 expression for cell entry into mitosis. WY-14,643 treatment also induced cholestasis and liver injury, which is evidenced by induction of alanine aminotransferase, alkaline phosphatase, and hepatic bile acid levels in WT mice. Hepatocyte RXRα deficiency protected the mice from WY-14,643-induced liver injury. WY-14,643-mediated induction of the small heterodimer partner, Mrp3, and Cyp3a11 levels was greater in hepatocyte RXRα-null than in WT mouse livers suggesting enhanced repression of bile acid synthesis and increased efflux of bile acids into blood for renal excretion as well as hydroxylation of bile acids because of hepatocyte RXRα deficiency. These data establish a crucial role of hepatocyte RXRα in regulating WY-14,643-mediated cell cycle progression as well as bile acid homeostasis.

Peroxisome proliferators (PPs), including the hypolipidemic fibrate drugs, are structurally and chemically diverse compounds that cause proliferation of hepatic peroxisomes, hyperplasia of hepatocytes, hepatomegaly, and induction of fatty acid oxidation gene expression (1–4). WY-14,643 is a potent peroxisome proliferator-activated receptor α (PPARα) ligand that has hypolipidemic properties and induces a 100% incidence of liver tumors in rats and mice (2, 4). The mechanisms underlying the carcinogenic effect of the PPs are not fully understood but do require the nuclear receptor PPARα (5–7).

Activation of PPARα by agonists occurs in both rodents and humans, but there are species differences in response to these PPs. Activation of PPARα is the basis for using gemfibrozil and fenofibrate to treat dyslipidemia (8). Chronic administration of PPs to mice and rats results in hepatocellular carcinomas; however, humans are resistant to PP-induced peroxisome proliferation and hepatocarcinogenesis. However, liver injury and acute cholestasis have been reported in humans because of administration of PPs (9–11). Reports also indicate that bile acids such as cholic acid and deoxycholic acid can serve as tumor promoters, suggesting that PP-induced neoplasia may involve alteration in bile acid homeostasis (12, 13).

Feeding the less specific PPARα agonist bezafibrate for 1 year resulted in hepatic cholestasis accompanied by significant suppression of Cyp7a1 mRNA levels in PPARα-null but not in wild type (WT) mice (14). An interpretation of these findings suggests that cholestasis associated with PPs may involve both PPARα-dependent and -independent mechanisms. Because PPARα heterodimerizes with retinoid X receptor (RXR) (15), studying the role of hepatocyte RXRα in PP-mediated cholestasis will indirectly prove that the effect is dependent or independent of PPARα.

Among the three RXR isoforms (α, β, and γ), RXRα is predominantly expressed in the liver (16). Our previous study demonstrated that WY-14,643-induced hepatocyte peroxisome proliferation was preserved in the absence of hepatocyte RXRα. WY-14,643-induced hepatomegaly was partially inhibited by RXRα deficiency, indicating the importance of RXRα in mediating WY-14,643-induced hepatomegaly and cholestasis. WY-14,643-mediated cell cycle progression and bile acid homeostasis were also partially inhibited by RXRα deficiency, suggesting that RXRα plays a crucial role in mediating WY-14,643-induced cell cycle progression and bile acid homeostasis.

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2 The abbreviations used are: PP, peroxisome proliferator; PPARα, peroxisome proliferator-activated receptor α; ALP, alkaline phosphatase; WT, wild type; ACOX1, acyl-CoA oxidase; RXR, retinoid X receptor; H-RXRα-null, hepatocyte RXRα-deficient; CDKs, cyclin-dependent kinase; ALT, alanine aminotransferase; FXR, farnesoid X receptor; SHP, small heterodimer partner; WY-14,643, 4-chloro-6-(2,3-sylidine)-pyrimidinylthio) acetic acid; Cdc2, cell cycle division cycle 2; PCNA, proliferating cell nuclear antigen; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Tnct, sodium/taurocholate-cotransporting polypeptide; IL-1β, interleukin-1β.
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in hepatocyte RXRα-deficient (H-RXRα-null) mice (17). It is not known whether the diminished hepatomegaly in H-RXRα-null mice involves resistance to alterations in cyclins and cyclin-dependent kinases (CDKs) that regulate the transit of cells through the cell cycle.

The goal of this study was as follows: 1) to examine the mechanism of WY-14,643-induced hepatocyte proliferation; 2) to study the role of hepatocyte RXRα in WY-14,643-induced hepatocyte proliferation; 3) to study the mechanism by which WY-14,643 causes cholestasis; and 4) to examine the effect of hepatocyte RXRα in WY-14,643-induced cholestasis. Accordingly, WT and H-RXRα-null mice were fed WY-14,643 for 2 weeks, and subsequent changes in hepatocyte proliferation and bile acid homeostasis were studied. Our data indicate that RXRα/PPARα is critical for regulating Wee 1-mediated Cdc2/cyclin B1 expression for cells to enter into mitosis. WY-14,643 induced cholestasis and liver injury in WT mice, as evidenced by elevated serum and hepatic bile acids, serum alkaline phosphatase (ALP), and alanine aminotransferase (ALT) levels, and marked inhibition of hepatic uptake transporters. In addition, hepatocyte RXRα deficiency resulted in marked induction of mRNA levels of small heterodimer partner (SHP), involved in repression of bile acid synthesis, Mrp3 (multidrug resistance-associated protein 3), involved in efflux of bile acids into blood for renal excretion, and Cyp3a11 that is involved in the hydroxylation of bile acids. Thus, H-RXRα-null mice were protected against WY-induced hepatotoxicity. Our present study with H-RXRα-null mice is the first to directly demonstrate the involvement of RXRα in WY-14,643-induced liver injury.

EXPERIMENTAL PROCEDURES

Animals—H-RXRα-null mice have been described previously (18). Age-matched WT (of mixed genetic background of C57Bl/6, 129/SvEvTac, and DBA-2) (18, 19) and H-RXRα-null mice (10–12 weeks old) were used in all the experiments. The mice were housed in steel microisolator cages at 22 °C with a 12:12-h, light/dark cycle. Mice were given a liquid diet. After 2 days of feeding mice with a control liquid diet, mice were randomized into two dietary groups (n = 5) and fed a diet containing 0.1% (w/v) 4-chloro-6-(2,3-sylidine)-pyrimidinylthio)acetic acid (WY-14,643) (EaglePicher Pharmaceutical Services, LLC, Lenexa, KS) or a control diet containing 0.0% WY-14,643. The 1-Amino Acid Defined AIN-93G Control Liquid Diet was used and purchased from DYETS Inc. (Bethlehem, PA). Individual body weights were recorded at the start and once a week thereafter. All procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. After 2 weeks of feeding control and WY-14,643 diets, blood samples were obtained and centrifuged at 3,000 rpm for 15 min to collect serum. Livers were rapidly excised, weighed, and portions snap-frozen in liquid nitrogen and maintained at −80 °C and later used for RNA or bile acid extraction and liver homogenate preparation. A portion of each liver was fixed in 10% formalin for hematoxylin and eosin staining.

Hematoxylin and Eosin Staining of Liver Sections—Liver specimens were fixed in 10% formalin/phosphate-buffered saline and then sliced and stained with hematoxylin and eosin for histological examination.

Quantification of Hepatic Bile Acids—Bile acids were extracted as described previously (20). Briefly, liver tissue (100 mg) was homogenized in t-butyl alcohol/double distilled H2O (1:1) and shaken on a rotator shaker overnight at room temperature. Following centrifugation at 5,000 rpm for 10 min, bile acid concentration in the supernatant was determined by a commercially available kit (colorimetric total bile acids assay kit, Bioquant, San Diego). Serum triglyceride levels were determined by triglyceride E-test kit (Wako Pure Chemical Industries, Richmond, VA).

Western Blot Analysis—Liver homogenates (50 μg/lane) were separated on 10 or 15% SDS-polyacrylamide gels, electroblotted onto polyvinylidene difluoride membrane, and immunoblotted with ACOXI (ABGENT, San Diego), cyclin D1, cyclin D3, Bcl-xl, and caspase 3 (Cell Signaling Technology, Boston), cell division cycle 2 (Cdc2), proliferating cell nuclear antigen (PCNA), or Wee 1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with the appropriate peroxidase-conjugated anti-rabbit or mouse IgG (Santa Cruz Biotechnology) secondary antibodies diluted in Tris-buffered saline with 0.1% Tween 20 (TBST) plus 1% nonfat dry milk for 1 h at room temperature. Following probing, blots were stripped and reprobed with anti-glyceraldehyde 3-phosphate dehydrogenase (Gapdh) antibody (Abcam Inc., Cambridge, MA). Proteins were viewed using enhanced chemiluminescence. Protein contents were determined by the Bradford method (21).

Determination of mRNA Expression Levels Using Real Time Quantitative Reverse Transcription-PCR—Total RNA was isolated from frozen liver tissues using the TRIzol reagent according to the manufacturer’s protocol (Invitrogen). RNA concentration and quality were determined spectrophotometrically at 260 and by the A260/A280 ratio, respectively. Real time quantitative PCR by Taqman or Sybr Green assay was performed to quantify the mRNA levels of ACOXI, Cyp3a11, Cyp7a1 (cholesterol 7α-hydroxylase), Cyp8b1 (sterol 12α-hydroxylase), cyclin D1, CDK2, cyclin A2, cyclin B1, Cdc2, SHP, interleukin-1β (IL-1β), Oatp1a1 (organic anion-transporting polypeptide 1a1), Oatp1a4, the Ntcp, Mrp3, and Gapdh. The Taqman reverse transcription-PCR was performed as described previously (22). Power Sybr Green PCR master mix (Applied Biosystems, Warrington, UK) was used for the Sybr Green assay. Each reaction contained 4 μl of cDNA diluted in water, 0.6 μl of each primer pair stock solution, 4.8 μl of water, and 10 μl of Sybr green reagent. Primer pairs were added to a final concentration of 5 μM in a total volume of 20 μl. The amplification reactions were carried out in the ABI 7900 real time PCR instrument.
(Applied Biosystems, Foster City, CA). Melting curve analysis was performed to ensure that the primer pairs amplified a single transcript. The comparative CT method was used to determine fold differences between samples. Primers and probes (Table 1) were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA).

**Statistical Analysis**—Data are presented as means ± S.E. (n = 4–5). Statistical analysis was performed using one-way analysis of variance followed by post hoc least significant difference (LSD) test. A p value of <0.05 was considered statistically significant.

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### TABLE 1
Sequences of primers and probes used for real time quantitative PCR

| Name       | Sequence                          | GenBank™ accession no. |
|------------|-----------------------------------|------------------------|
| Cyclin D1  | Sense: CTGCGCTCTCTAGAAGTGAAGCA    | NM007631               |
|            | Antisense: CTCGGCGGARATACCTTCTCCT |                        |
|            | Probe: GCCCGCTCTCAGCAAGGCTTCAG    |                        |
| Cdk2       | Sense: TCCCTCACTAGAAGTGAAGCAA     | NM183417               |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
|            | Probe: GTGCGCCTCTCAGCAAGGCTTCAG  |                        |
| Cyclin B1  | Sense: GACAGATGGGAGTGAAGTGAAGCA  | NM172301               |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
|            | Probe: GTGCGCCTCTCAGCAAGGCTTCAG  |                        |
| Cyclin A2  | Sense: GACAGATGGGAGTGAAGTGAAGCA  | X75483                 |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
|            | Probe: GTGCGCCTCTCAGCAAGGCTTCAG  |                        |
| Cdc2       | Sense: TGTATCAGGAAGAATAGTTTCAG    | M38724                 |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
|            | Probe: GTGCGCCTCTCAGCAAGGCTTCAG  |                        |
| Acox1      | Sense: TCGCTTACTCCAGCTCTGTCCAG   | NM015729               |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
|            | Probe: GTGCGCCTCTCAGCAAGGCTTCAG  |                        |
| Cyp3a11    | Sense: TACAGACCCAGAAGAAGTTAAGCA  | NM007818               |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
|            | Probe: GTGCGCCTCTCAGCAAGGCTTCAG  |                        |
| Mrp3       | Sense: CACATCTTACAAAACCTCTCCAT   | NM029600               |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
|            | Probe: GTGCGCCTCTCAGCAAGGCTTCAG  |                        |
| Cyp8b1     | Sense: GACCCTTACAAACCTCTCCATT    | NM007824               |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
|            | Probe: GTGCGCCTCTCAGCAAGGCTTCAG  |                        |
| IL-1β      | Sense: AAAGATGAGGGCTGCTTTCCA    | NM008361               |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
|            | Probe: GTGCGCCTCTCAGCAAGGCTTCAG  |                        |
| SHP         | Sense: AGACACCTCTCTCTGTTAGCCT    | NM011850               |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
|            | Probe: GTGCGCCTCTCAGCAAGGCTTCAG  |                        |
| Ntcp       | Sense: TCTCCCTCTGAGTCTCCACTC    | NM013797               |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
|            | Probe: GTGCGCCTCTCAGCAAGGCTTCAG  |                        |
| Oatp1a1a   | Sense: AATACCTGAGGACACATATXGA    | AR031814               |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |
| Oatp1a4a   | Sense: AATACCTGAGGACACATATXGA    | AR031814               |
|            | Antisense: AAGTTTGAGGGCTTCTCTTTGA |                        |

*SHP, Ntcp, Oatp1a1, and Oatp1a4 real time PCR was performed using Sybr Green assay.

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**RESULTS**

**WY-14,643-induced Hepatomegaly and Mitosis Were Inhibited in H-RXRα-Null Mice**—WY-14,643 ingestion decreased body weight in both the WT and H-RXRα-null mice (Fig. 1a). However, the decrease in body weight was greater in WT than in H-RXRα-null mice (Fig. 1a). In WT mice, this effect was...
accompanied by increases in absolute liver weight (2.1-fold) (Fig. 1b) and liver-to-body weight ratio (2.5-fold) (Fig. 1c). WY-14,643-induced increases in absolute liver weight and liver/body weight were drastically reduced by hepatocyteRXRα deficiency (Fig. 1, b and c). Treatment of WT and H-RXRα-null mice with WY-14,643 resulted in enlarged cells in both genotypes (Fig. 2). Features of liver pathology, including mitotic cells (arrowheads), were common in WT mice fed WY-14,643 but scanty in WY-14,643-fed H-RXRα-null mice.

Effect of WY-14,643 and RXRα Deficiency on Hepatic Cell Cycle-related Gene and Protein Expression—We next studied the possibility that factors that favor hepatocyte proliferation and/or inhibition of apoptosis may account for the liver size increases seen in WY-14,643-treated WT but not in H-RXRα-null mouse livers. The expression of cell cycle genes was examined. Cyclins and CDKs regulate the molecular events of cell division, and overexpression of these proteins causes uncontrolled cell growth. WY-14,643 treatment increased cyclin D1 mRNA levels in both WT (2.8-fold) and H-RXRα-null (1.8-fold) mice (Fig. 3a). Levels of cyclin D1 and cyclin D3 protein were examined by Western blot. WY-14,643 increased cyclin D1 and cyclin D3 protein levels by 4.0- and 2.4-fold, respectively, in WT mice (Fig. 3f and g). However, such an induction was not found in WY-14,643-fed RXRα-null mice (Fig. 3f and g). CDK2 mRNA levels were unchanged in both genotypes after WY-14,643 feeding (Fig. 3b). Furthermore, WY-14,643 increased cyclin A2 mRNA levels only in WT mice (15.1-fold) (Fig. 3c). The basal cyclin B1 mRNA levels were lower in the H-RXRα-null mice than in WT mice (Fig. 3d). WY-14,643 increased cyclin B1 mRNA levels in WT and H-RXRα-null mice by 10.2- and 3.7-fold, respectively (Fig. 3d). Constitutive Cdc2 mRNA and protein levels were lower in the H-RXRα-null mice than in WT mice (Fig. 3e and h). Furthermore, WY-14,643 increased levels of Cdc2 mRNA (3.6-fold) and protein (4-fold) only in WT mice (Fig. 3e and h) and not in knock-out mice. Entry into mitosis is regulated by the balance between the opposing activities of Cdc2 and Wee 1. Down-regulation of Wee 1 allows activation of Cdc2/cyclin B kinase during the G2/M transition (23). In agreement with the increased hepatic Cdc2 protein levels in WY-14,643-fed WT mice, protein levels of Wee 1 were decreased in WY-14,643-fed WT mouse livers (Fig. 3i) (23). Wee 1 protein levels seemed to be reduced by WY-14,643 in H-RXRα-null mouse livers, but they did not reach statistical significant difference (Fig. 3i). Furthermore, Wee 1 protein levels in WY-14,643-fed H-RXRα-null mouse livers were much higher than those in WY-14,643-fed WT mouse livers (Fig. 3i). PCNA has been demonstrated as a marker of hepatocyte proliferation (24). PCNA protein levels were markedly induced in WY-14,643-fed WT mice (Fig. 3j); however, such induction was not apparent in H-RXRα-null mouse livers (Fig. 3j). These observations indicate that RXRα/PPARα is critical for regulating both the G2/M interface for cell cycle initiation and for the Wee 1-mediated Cdc2/cyclin B1 expression for entry into mitosis.

Effect of WY-14,643 and RXRα Deficiency on Apoptotic, Cytokine, and Peroxisomal Gene Expression—PPs induce hepatocyte proliferation and suppress apoptosis leading to liver enlargement (25). Furthermore, the involvement of cytokines, including IL-1, in PP-mediated hepatocyte growth and apoptosis suppression has been suggested (26, 27). We therefore examined whether WY-14,643 regulates the expression of pro- and anti-apoptotic proteins as well as IL-1β gene expression. The basal Bcl-xL protein levels were weakly induced because of hepatocyteRXRα deficiency, but it did not reach statistical significance, confirming our previous finding (Fig. 4a) (22). WY-14,643 did not affect Bcl-xL protein levels either in WT or in H-RXRα-null mouse livers (Fig. 4a). The basal procaspase 3 protein levels were not different between WT and H-RXRα-null mice (Fig. 4b). Furthermore, no procaspase 3 cleavage products were observed in either WT or H-RXRα-null mice after WY-14,643 treatment (Fig. 4b). Thus, it is unlikely that apoptosis is affected because of WY-14,643 treatment or RXRα deficiency.

WY-14,643 treatment induced the levels of IL-1β mRNA in the WT mice (2.1-fold), whereas no increase was seen in H-RXRα-null mice (Fig. 4c). ACOX1 is localized in the peroxisomes, and its induction is a specific marker of peroxisome proliferation (28). Constitutive ACOX1 mRNA and protein levels were lower in the H-RXRα-null mice than in WT mice (Fig. 4d). WY-14,643 treatment increased ACOX1 mRNA as well as protein levels in both genotypes; however, the levels remained lower in RXRα-null mouse livers (Fig. 4d).
increased 2.8-fold in WT mice (Fig. 5c) by WY-14,643, whereas no increase was seen in H-RXRα-null mice (Fig. 5c). Serum triglycerides levels were higher in H-RXRα-null mice (4.2-fold) than in WT mice (Fig. 5d). WY-14,643 decreased serum triglyceride levels in both genotypes of mice (Fig. 5d). Basal hepatic bile acid levels were higher in H-RXRα-null mice than in WT mice (Fig. 5e). Hepatic bile acid levels were increased by WY-14,643 in WT mice (2.8-fold), but no increase was seen in H-RXRα-null mice (Fig. 5e).

**Effect of WY-14,643 and RXRα Deficiency on mRNA Levels of Cyp7a1, Cyp8b1, and SHP**—Cyp7a1 catalyzes the rate-limiting step in bile acid formation from cholesterol (29). Cyp8b1 is required for the synthesis of cholic acid, a major bile acid that accumulates during cholestasis (29, 30). Cyp7a1 gene expression was not affected by WY-14,643 treatment in WT mice (Fig. 6a). RXRα deficiency increased Cyp7a1 mRNA levels (1.6-fold), but it did not reach statistical significance in the current experiment (Fig. 6a). However, our previous report demonstrated the induction of Cyp7a1 mRNA levels because of hepatocyte RXRα deficiency (18). Although WY-14,643 treatment tended to decrease mRNA levels of Cyp7a1 in H-RXRα-null mice, the decrease was not statistically significant (Fig. 6a). Interestingly, Cyp8b1 was decreased by WY-14,643 in both WT and H-RXRα-null mice (Fig. 6b). The basal SHP mRNA levels were lower in the H-RXRα-null mice than in WT mice (Fig. 6c). SHP gene expression was not affected by WY-14,643 treatment in WT mice (Fig. 6c). However, WY-14,643 increased SHP mRNA levels in H-RXRα-null mice (2-fold) (Fig. 6c).

**Effect of WY-14,643 and RXRα Deficiency on mRNA Levels and Protein Expression of Hepatic Bile Acid Uptake and Efflux Transporters and Detoxification Genes**—Bile acid-induced liver damage may be counteracted by decreased uptake or increased export of bile acids into blood leading to decreased exposure of the liver to bile acids during cholestasis. A number of bile acid transport proteins are involved in this process. We therefore examined the expression of both hepatic uptake and efflux transporters. WY-14,643 ingestion decreased gene expression of Ntcp, which is responsible for the uptake of bile acids from
Portal blood into hepatocytes, only in WT mice (Fig. 7a). The Oatps are a group of membrane carriers critically involved in sodium-independent transport of various amphipathic organic compounds, including bile acids into hepatocytes (31). Furthermore, mRNA level of uptake transporter Oatp1a1 was completely inhibited in WT mice fed WY-14,643 (Fig. 7b). However, in H-RXRα-null mice, WY-14,643 inhibited Oatp1a1 mRNA levels by only 58% (Fig. 7b). WY-14,643 decreased the mRNA levels of the hepatic uptake transporter Oatp1a4 by 81 and 74% in WT and H-RXRα-null mice, respectively (Fig. 7c).

The expression of basolateral efflux transporter Mrp3 mRNA was also quantified (Fig. 7d). The basal Mrp3 expression was much lower in the H-RXRα-null mice than in the WT mice, as reported previously (32). WY-14,643 increased Mrp3 gene expression in WT (2.6-fold) and in H-RXRα-null (3.6-fold) mouse livers (Fig. 7d). Bile acid detoxification can occur via hydroxylation mediated by Cyp3a11. WY-14,643 treatment increased hepatic Cyp3a11 mRNA levels in WT (1.7-fold) and in H-RXRα-null (3.9-fold) mice (Fig. 8a). The basal Cyp3a11 protein levels were lower in the H-RXRα-null mice than in WT mice (Fig. 8b). Furthermore, in agreement with induction of Cyp3a11 gene expression, Cyp3a11 protein levels were increased by WY-14,643 in WT (1.9-fold) and in H-RXRα-null (3.1-fold) mice (Fig. 8b). Our data indicate that WY-14,643 can coordinately induce bile acid efflux transporter and detoxification enzymes, which are similar to that seen after bile duct ligation and cholic acid-challenged animal models (33).

**DISCUSSION**

This study demonstrates that WY-14,643 administration causes decreased body weight, induced hepatomegaly, and increased hepatocyte proliferation in WT mice, whereas these changes were minimal in H-RXRα-null mice. We also show that the expression of cell cycle-related genes cyclin D1, D3, A2, B1, and Cdc2 were markedly increased in WY-14,643-fed WT mouse liver, which is consistent with a previous report (44). In the WY-14,643-fed H-RXRα-null mouse livers, cyclins D1 and B1 mRNA levels were increased; however, both cyclin D1 and D3 protein levels were not...
increased. Cyclins and CDKs regulate the molecular events of cell division, and overexpression of these proteins causes uncontrolled cell growth.

Cdc2, also known as Cdk1, is an initiator of mitosis and serves as a marker for cellular proliferation (34, 35). The protein levels of Cdc2 and Wee 1 were significantly altered by WY-14,643 in WT but not in H-RXRα-null mouse livers. Wee 1 inactivates Cdc2 by phosphorylation of Cdc2 on Tyr-15 to inhibit mitosis (23). WY-14,643-induced down-regulation of Wee 1 expression should lead to reduction of Cdc2 phosphorylation. Thus, our results suggest that WY-14,643 not only increases the expression of Cdc2 but also inhibits the Wee 1-Cdc2 pathway-mediated hepatocyte proliferation in WT mice. Interestingly, the basal expression of cyclin B1 and Cdc2 were reduced because of hepatocyte RXRα deficiency, indicating that these cell cycle regulatory genes are controlled by RXRα. Previous reports have demonstrated a correlation between cyclin A mRNA levels and the expression of RXRα mRNA in Hep3B cells, implicating a role for RXRα in enhancing cell proliferation and promoting cell cycle progression (36). Our data also indicate that PCNA protein, a marker for cell proliferation, was increased by WY-14,643 in WT mouse livers but not in H-RXRα-null mouse livers. Overall, our data indicate that hepatocyte RXRα plays a crucial role in cell proliferation and that Wee 1-Cdc2 pathway-mediated hepatocyte proliferation is tightly controlled by PPARα/RXRα.
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Figure 7. Hepatic Ntcp, Oatp1a1, Oatp1a4, and Mrp3 mRNA levels in WT and H-RXRα-null mice fed control or WY-14,643 diet. Male WT or H-RXRα-null mice were fed control (■) or WY-14,643 (■) diets for 2 weeks. Total hepatic RNA was used to study gene expression by real time PCR. Data represent mean ± S.E. (n = 5). Asterisks represent statistically significant differences (p < 0.05) between WT and H-RXRα-null mice. Pound signs represent statistically significant differences (p < 0.05) between mice fed control and WY-14,643 diets. Daggers represent statistically significant differences (p < 0.05) between WT mice and H-RXRα-null mice fed WY-14,643 diets.

Figure 8. Hepatic gene and protein expression of Cyp3a11 in WT and H-RXRα-null mice fed control or WY-14,643 diet. Male WT or H-RXRα-null mice were fed control (■) or WY-14,643 (■) diets for 2 weeks. Total hepatic RNA was used to study gene expression by real time PCR. Liver homogenate (50 μg/lane) from control or WY-14,643-fed mice was used for Western blot analysis by probing with anti-Cyp3a11 antibody. Anti-Gapdh antibody was used as a loading control. Data represent mean ± S.E. (n = 3–5). Pound signs represent statistically significant differences (p < 0.05) between mice fed control and WY-14,643 diets. Daggers represent statistically significant differences (p < 0.05) between WT mice and H-RXRα-null mice fed WY-14,643 diets.

IL-1β mRNA levels were increased after WY-14,643 ingestion only in WT mice, raising the possibility that IL-1β may be involved in priming WT mice hepatocytes for cell cycle entry. Furthermore, a role of IL-1β in induction of DNA synthesis and inhibition of apoptosis after PP treatment has been demonstrated in vitro (27). We therefore looked for evidence of the cleaved procaspase 3 and of changes in protein levels of anti-apoptotic Bcl-xL. Neither procaspase 3 cleavage products nor changes in Bcl-xL levels were observed in WT or H-RXRα-null mice fed WY-14,643. Thus, our findings indicate that the hepatomegaly observed in WY-14,643-fed WT mice was entirely because of an increase of hepatocyte proliferation but not because of inhibition of apoptosis. In addition, inhibition of cell cycle progression, but not enhanced apoptosis, was a major mechanism for the resistance against WY-14,643-induced liver cell growth in H-RXRα-null mice.

A differential hepatotoxic phenotype in response to WY-14,643 was also observed in WT and H-RXRα-null livers. A marked increase in serum ALT, ALP, and bile acids levels was seen in WY-14,643-fed WT mouse livers, whereas only a slight increase in ALP was seen in WY-14,643-fed H-RXRα-null mouse livers, with no increase in ALT or bile acids levels. Furthermore, WY-14,643 increased hepatic bile acid levels only in WT mice. This increase in bile acid levels can be explained by a marked inhibition of the major Na⁺-dependent bile acid transporter, Ntcp, and complete repression of the Oatp1a1 transporter in WT mice fed WY-14,643. Ntcp and Oatp1a1 are down-regulated after bile duct ligation or lipopolysaccharide administration, both of which are used as experimental rodent cholestasis models (37). Previous reports indicate that the less specific PPARα agonist benzafibrate, but not WY-14,643, induced cholestasis in PPARα-null mice, suggesting that specific effect of PXIs is because of the relative hepatic levels of RXRα and PPARα in different species remains to be examined.

Cyp7a1, the key enzyme mediating the rate-limiting step in bile acid synthesis, was not affected by WY-14,643 in mice of
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either genotype. However, Cyp8b1, involved in cholic acid synthesis, was repressed by WY-14,643 in both WT and in H-RXRα-null mice. The repression of Cyp8b1 and increase in hepatic bile acid levels observed in WY-14,643-fed WT mice suggest that WY-14,643 induces bile acid synthesis and alters bile acid homeostasis. Our data are consistent with a previous report, which shows that WY-14,643 increases cholic acid levels (41). Cholod acid administration suppresses Cyp8b1 by negative feedback regulation of bile acid biosynthesis (42). Our data showed suppression of Cyp8b1, which should decrease bile acid levels induced by WY-14,643.

This current study as well as previous data suggest that loss of hepatocyte RXRα has a major impact on genes regulated by farnesoid X receptor (FXR) but not on genes regulated by liver X receptor (43). For instance, the basal gene expression of SREBP-1c (sterol regulatory element-binding protein 1c), which is regulated by liver X receptor, is not changed in H-RXRα-null mice compared with WT mice (43). Furthermore, hepatocyte RXRα deficiency did not change basal mRNA levels of ACC-1a (acyetyl-CoA carboxylase-1a) or fatty-acid synthase, target genes for SREBP-1c in H-RXRα-null mouse livers (43). Interestingly, loss of hepatocyte RXRα resulted in a decrease in gene expression of SHP, which is important for FXR-dependent bile acid negative feedback regulation (44). By Northern blot analysis, our previous studies showed that Cyp7a1 mRNA levels were elevated because of hepatocyte RXRα deficiency (18). However, in this study, the basal Cyp7a1 mRNA levels were only modestly increased in H-RXRα-null mice compared with WT mice. Cyp7a1 is a circadian gene (29). The variation in fold changes could be due to when the mice were sacrificed. In this study and in our previous reports, Mrp3, which is important for the export of bile acids out of the liver, and Mdr2 (multiple drug resistance protein 2), which transports phospholipids into bile, are significantly decreased in H-RXRα-null mice (32, 43). These changes suggest that hepatocyte RXRα deficiency may have an impact on the maintenance of bile acid homeostasis. It is therefore plausible that changes observed in SHP, Cyp7a1, Mrp3, and Mdr2 gene expression because of hepatocyte RXRα deficiency may affect FXR activity and account for the increase in hepatic bile acid levels in H-RXRα-null mice seen in this study.

Enhanced bile acid hydroxylation by Cyp3a11 in FXR-null mice contributed to resistance against bile acid-induced toxicity (33). Furthermore, elevated Mrp3 expression observed in patients with cholestasis and in pregnant X receptor-null mice administered cholic acid has been linked to protection against liver injury (20, 45). In our model, the WY-14,643-mediated induction of Cyp3a11, Mrp3, and SHP levels was greater in H-RXRα-null than WT mouse livers. These data indicate that Cyp3a11-mediated bile acid hydroxylation, Mrp3-mediated basolateral efflux of bile acids into blood for renal excretion, and SHP-mediated suppression of bile acid synthesis protected H-RXRα-null mice against WY-14,643-induced cholestasis and hepatotoxicity. The basal Cyp3a11 level was reduced because of hepatocyte RXRα deficiency; however, the inducibility of Cyp3a11 remained in the mutant mice, which is consistent with our previous observation that Cyp3a11 induction by 5-pregn-3β-ol-20-one-16α-carbonitrile occurs in the livers of H-RXRα-null mice (46).

In conclusion, we show the protective effect of hepatocyte RXRα deficiency on WY-14,643-induced hepatocyte proliferation as well as cholestatic liver injury. WY-14,643 not only regulates cell cycle progression but also bile acid homeostasis. This study provides important new insights into the molecular mechanisms of the toxic effects of PPs and the role of hepatocyte RXRα in these actions. These observations may have relevance for the pathophysiology of hepatocyte proliferation and cholestatic liver disease. Further studies are required to determine whether PPARα-null mice are protected against WY-14,643-induced hepatocarcinogenesis and cholestatic liver fibrosis.

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