Chronic Exposure to Rifaximin Causes Hepatic Steatosis in Pregnane X Receptor-Humanized Mice

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Rifaximin, a nonsystemic antibiotic that exhibits low gastrointestinal absorption, is a potent agonist of human pregnane X receptor (PXR), which contributes to its therapeutic efficacy in inflammatory bowel disease. To investigate the effects of long-term administration of rifaximin on the liver, PXR-humanized mice were administered rifaximin for 6 months; wild-type and *Pxr*-null mice were treated in parallel as controls. Histological analysis revealed time-dependent intense hepatocellular fatty degeneration and increased hepatic triglycerides in PXR-humanized mice and not in wild-type and *Pxr*-null mice. After long-term treatment, PXR target genes were induced in small intestine and liver, with significant up-regulation in the expression of hepatic genes related to triglyceride synthesis and lipid accumulation. However, no significant hepatic accumulation of rifaximin was found, even after 6 months of treatment, in PXR-humanized mice. Genes in the small intestine that are involved in the uptake of fatty acids and triglycerides were induced along with increased triglyceride accumulation in intestinal epithelial cells of PXR-humanized mice; this was not observed in wild-type and *Pxr*-null mice. These findings suggest that long-term administration of rifaximin could lead to PXR-dependent hepatocellular fatty degeneration as a result of activation of genes involved in lipid uptake, thus indicating a potential adverse effect of rifaximin on liver function after long-term exposure.

Key words: rifaximin; hepatotoxicity; human pregnane X receptor; steatosis.

Rifaximin (Xifaxan), a nonsynthetic antibiotic that has low gastrointestinal absorption while retaining antibacterial activity within the intestine (Ojetti *et al.*, 2009), was approved in 2004 for therapy of traveler's diarrhea and in 2011 for therapy of hepatic encephalopathy (HE) due to its antibiotic-based inhibition of ammonia-producing enteric bacteria that reduces circulating gut-derived ammonia in patients with cirrhosis (Khan *et al.*, 2011). In addition, a large clinical trial revealed that rifaximin has efficacy toward irritable bowel syndrome (IBS) presumably as a result of alteration of intestinal microbiota (Pimentel, 2009). Thus, rifaximin has therapeutic applications beyond its antibiotic activity and is especially attractive due to its minimal systemic absorption and safety profile. Rifaximin is

a gut-specific agonist for the human pregnane X receptor (PXR) and is a potential drug candidate for IBS and potentially inflammatory bowel disease (IBD) through its anti-inflammatory activity due in part to inhibition of the NF- κ B signaling cascade (Cheng *et al.*, 2010; 2012). However, despite the established function and mechanism of rifaximin on human PXR after short-term administration, the PXR-dependent safety and efficacy of this drug after long-term treatment, which is required for IBS and IBD therapy (Triantafyllidis *et al.*, 2011), remains to be investigated in an animal model.

PXR is a ligand-activated transcription factor and a member of the nuclear receptor superfamily (Zhang *et al.*, 2008). Ligands of PXR are structurally diverse and display species specificity; for example, rifampicin is an agonist for human PXR but does not activate rodent PXR, in contrast to pregnenolone-16- α -carbonitrile (PCN), a rodent-specific PXR agonist that does not activate human PXR (Cheng *et al.*, 2009). Activated PXR is involved in the control of drug metabolism, through its regulation of genes involved in the transport, metabolism, and elimination of drugs and other foreign compounds. Recent studies revealed that PXR is emerging as a pivotal nuclear receptor involved in the regulation of endogenous metabolic homeostasis, including anti-inflammation, controlling lipid, bile acid, and steroid hormone homeostasis (Schote *et al.*, 2007). It was reported that PXR and other nuclear receptors induce expression of cluster of differentiation 36 (CD36), a plasma membrane-bound transporter that contributes to fatty acid uptake and contributes to control of the sterol regulatory element-binding protein (SREBP)-independent lipogenic pathway (Zhou *et al.*, 2008). Thus, PXR could be involved in lipid homeostasis. In the current study, PXR-humanized mice coupled with wild-type and *Pxr*-null controls were applied to evaluate the potential function of long-treatment rifaximin on liver function and to provide experimental evidence for the clinical safety and toxicity of rifaximin.

MATERIALS AND METHODS

Animals and chemicals. PXR-humanized (hPXR), wild-type (WT), and *Pxr*-null male mice were housed in temperature- and light-controlled rooms and were given water and pelleted chow ad libitum. All animal experiments

were carried out in accordance with the Institute of Laboratory Animal Resources Guidelines and approved by the National Cancer Institute Animal Care and Use Committee. Rifaximin was provided by Salix Pharmaceuticals, Inc. (Morrisville, NC).

Experimental design. Two- to three-month-old hPXR, WT, and *Pxr*-null male mice were fed AIN-93G purified diets containing rifaximin (10 mg/kg), which is equivalent to the oral administration of 1 mg/kg/day to each mouse. Serum and tissue samples were collected by retro-orbital bleeding, and mice were killed by CO₂ asphyxiation after rifaximin administration for 1 week, 1 month, 3 months, and 6 months. Control groups of hPXR, WT, and *Pxr*-null mice were fed the AIN-93G diet and killed at 6 months after treatment when serum and tissues were collected. Liver, small intestine, colon, and other tissue samples were harvested and stored at -80°C before analysis.

Assessment of liver injury. For assessment of macroscopic liver damage, liver tissue was flushed with PBS and fixed in 10% buffered formalin. Liver injury was scored by double-blinded analysis on a routine hematoxylin and eosin-stained section according to the morphological criteria previously described (Schulte, 1991). Drug-induced liver injury was further evaluated by measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum. Briefly, 2 µl of serum was mixed with 200 µl of AST or ALT assay buffer (Catachem, Bridgeport, CT) in a 96-well microplate, and the oxidation of NADH to NAD⁺ was monitored at 340 nm for 5 min.

Fatty acid uptake and AMP/ATP ratio measurements. Plasma levels of nonesterified free fatty acid, triglycerides, and total cholesterol in serum and liver homogenate were measured in overnight-fasted mice using assay kits from Wako Diagnostics (Wako Diagnostics, Richmond, VA). Liver homogenate was extracted as follows: 20 mg liver was homogenized with 200 µl extraction buffer (50 mM Tris/HCL, 1% Triton X-100 (vol/vol)). One microliter of supernatant was used for the measurements as described for serum analysis according to the manufacturer's protocol (Wako). To analyze AMP (adenosine monophosphate)/ATP (adenosine-5'-triphosphate) ratios in liver homogenates, 10 µl of supernatant used (ATP determination Kit, Invitrogen). AMP was quantitated by Xevo G2 QTOF (Waters) with 100 mg liver homogenized in 1 ml acetonitrile (100%, vol/vol, acetonitrile:H₂O, with internal standard). The solutions were centrifuged three times (14,300 × g, 15 min, 4°C), and the supernatants from the last centrifugation processed. The intestine was divided into three equal-length segments (S1–S3) from proximal to distal, washed with ice-cold phosphate-buffered saline, opened longitudinally, and epithelial cells were scraped and placed into PBS. Phenol/chloroform extracts containing the lipid contents were analyzed with nonesterified free fatty acid, triglycerides, and total cholesterol, respectively (Wako Diagnostics, Richmond, VA).

RNA analysis. Hepatic and intestinal RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and qPCR performed using cDNA generated from 1 µg of total RNA with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Primers for qPCR were designed using the Primer Express software (Applied Biosystems, Foster City, CA); sequences are available upon request. qPCR reactions were carried out using SYBR Green PCR Master Mix (SuperArray, Frederick, MD) by using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Values were quantitated using the comparative cycle threshold (CT) method, and results were normalized to mouse β-actin.

Protein analysis and IHC staining of Cd36 and Fabp2 in small intestine. Colon tissues were collected and epithelial cells isolated and extraction carried out by RIPA buffer as described (Peach *et al.*, 2012). Primary antibodies to Cd36 (Novus Biologicals, CO, USA) diluted 1:2000, Fabp2 (Novus Biologicals, CO, USA) diluted 1:10,000 with TBST, followed by peroxidase-conjugated anti-rabbit or anti-goat IgG diluted 1:10,000 with TBST were used for Western blot analysis, with β-actin used as a loading control. For immunohistochemical staining, the small intestine was opened longitudinally, flushed with PBS, and fixed in 10% buffered formalin. Paraffin-embedded slides were retrieved and incubated with primary Fabp2 at 1:1000 in background-reducing diluents for 1 h at room temperature. After washing, the tissue was incubated with horseradish peroxidase-labeled anti-goat antibody (Cell Signaling), and the blots were developed with betazoid diaminobenzidine (Biocare Medical) incubation

for 10 min at room temperature and then counterstained with hematoxylin for 5 min. For negative controls, the tissue sections were incubated without primary antibody in Tris-buffered saline and 1% bovine serum albumin. Digital images of jejunum from hPXR control and hPXR-treated mice were acquired at the same time under identical light and exposure conditions. Images of tissues from control and rifaximin-treated hPXR treated mice were grouped prior to any post-acquisition adjustment of contrast or brightness so that all images were adjusted identically.

Metabolomics analysis of urine, feces, serum and liver homogenate. Urine and feces were collected after rifaximin administration. Urine samples were processed by mixing 40 µl of urine with 160 µl of 50% aqueous acetonitrile and centrifuging at 18,000 × g for 10 min to remove protein and particulates. Feces (100 mg) were homogenized with 100% acetonitrile, and the supernatants after three time-centrifugations were collected and processed. Liver tissues (100 mg) from control, 1 week, 1 month, 3 months, and 6 months treatment with rifaximin were homogenized on ice, and acetonitrile (50% vol/vol water, 1 ml) was added for denaturation of protein. After multiple centrifugations, supernatants were collected. Serum samples were denatured by a 40-fold dilution with acetonitrile (66% vol/vol water), and supernatants were processed for liquid chromatography analysis. Supernatants were injected into a UPLC system (Waters Corporation, Milford, MA), and metabolites were separated by a gradient ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid over a 10-min run. An Acquity UPLC BEH C18 column (Waters) was used to separate chemical components at 35°C. The mobile phase flow rate was 0.5 ml/min with an aqueous acetonitrile gradient containing 0.1% formic acid over a 10-min run (0% acetonitrile for 0.5 min to 20% acetonitrile by 5 min to 95% acetonitrile by 9 min, and equilibrated at 100% water for 1 min before the next injection). The QTOF Premier mass spectrometer was operated in the positive electrospray ionization mode. Capillary voltage and cone voltage were maintained at 3 kV and 20 V, respectively. Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as both cone gas (50 l/h) and desolvation gas (600 l/h), and argon was used as collision gas. For accurate mass measurement, the time-of-flight mass spectrometer (TOFMS) was calibrated with sodium formate solution (range *m/z* 100–1000) and monitored by the intermittent injection of the lock mass sulfadimethoxine ([M+H]⁺ = 311.0814 *m/z*) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx software (Waters) in centroid format.

Principal component analysis of metabolomic data. Chromatographic and spectral data were deconvoluted by MarkerLynx software (Waters Corp.). A multivariate data matrix containing information on sample identity, ion identity (retention time and *m/z*), and ion abundance was generated through centroiding, deisotoping, filtering, peak recognition, and integration. The intensity of each ion was calculated by normalizing the single ion counts versus the total ion counts in the whole chromatogram. The data matrix was further exported into SIMCA-P software (Umetrics, Kinnelon, NJ) and transformed by mean-centering and Pareto scaling, a technique that increases the importance of low abundance ions without significant amplification of noise. Principal components of serum were generated by principal components analysis (PCA) to represent the major latent variables in the data matrix and were described in a scores scatter plot.

Statistics. Experimental values are expressed as mean ± standard deviation (SD). Statistical analysis was performed with two-tailed Student's *t* tests, with a *p* value of < 0.05 considered statistically significant.

RESULTS

Effect of Rifaximin on Hepatocellular Fatty Degeneration

Histological analysis revealed that hPXR mice treated for 6 months with rifaximin have significant vacuolated macrovesicles, in contrast to the normal hepatic architecture in similarly treated WT, *Pxr*-null mice, and untreated hPXR mice (Fig. 1A).

FIG. 1. (A) HE staining revealed time-dependent administration of rifaximin to hPXR mice demonstrated gradual enhancement of hepatocellular fatty degeneration without nodular hyperplasia. Upper-left: control, upper-right: 1-month treatment with rifaximin, below-left: 3-month treatment with rifaximin, and below-right: 6-month treatment of rifaximin. Magnification: $\times 200$. (B) ALT and AST shows no significant difference in hPXR mice treated with 1 week, 1 month, 3 month, and 6 months with rifaximin. However, serum triglycerides and serum free fatty acid reveal significant decrease when treated rifaximin for 1 month, 3 months, and 6 months when compared with control and 1 week administration in hPXR mice. $^*p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with 1 week, $^{\#\#}p < 0.01$ compared with 1 week; $^{\$}p < 0.05$ compared with 3 months.

Time-dependent administration of rifaximin to hPXR mice demonstrated a gradual enhancement of hepatocellular degeneration without nodular hyperplasia. Serum ALT and AST activities, sensitive diagnostic indicators of hepatotoxicity (Ozer *et al.*, 2008), were not significantly different between hPXR, WT, and *Pxr*-null mice after 1 week, 1 month, 3 months, and 6 months of rifaximin treatment (Fig. 1B). Serum triglycerides and nonesterified free fatty acids were significantly decreased after 1, 3, and 6 months of treatment when compared with control and 1 week administration, while serum cholesterol levels were not changed (Fig. 1B). Liver triglycerides were markedly increased by 5- and 10-fold compared with controls in hPXR mice treated with rifaximin for 3 and 6 months, despite lower ratios of liver *versus* body in mice treated for 3 and 6 months (Fig. 2). A slight decrease of hepatic cholesterol levels were noted after rifaximin treatment for 1, 3, and 6 months and a 50% decrease in liver free fatty acids observed only at 6 months of treatment. Additionally, mild fibrosis was found only in

hPXR mice treated for 6 months with rifaximin as revealed by Sirius Red staining (Tanaka *et al.*, 2012); this was not observed after short-term treatment of hPXR mice, and after long-term treatment of WT and *Pxr*-null mice (Supplementary fig. 1). Expression of genes encoding tumor necrosis factor β 1 (TNF β 1), collagen type 1 α 1 (Col1 α 1), and α -actin-2 (Acta2; Minguez and Lachenmayer, 2011) showed a small but significant increase after 6-month treatment with rifaximin in hPXR, and TNF β 1 was significantly increased compared with the controls and shorter time course administration of rifaximin to hPXR mice (Supplementary fig. 1).

In contrast to the response of hPXR mice, WT and *Pxr*-null mice reveal no significant histological change as well as change on serum/liver biochemistry, including serum or liver nonesterified free fatty acid, triglycerides, and total cholesterol (Supplementary fig. 2). Compared with the different response to rifaximin-mediated hepatic lipidosis between hPXR mice and WT and *Pxr*-null mice, these results suggested a human

FIG. 2. Liver triglycerides demonstrated a marked increase in liver from hPXR mice treated with rifaximin for 3 and 6 months (below left). A 5- and 10-fold increase in triglyceride compared with the triglyceride in control and 1-week rifaximin-treated hPXR mice, respectively, despite lower ratios of liver versus body in mice treated for 3 and 6 months (below right), while only small changes in liver free fatty acids (upper left) as well as liver cholesterol levels (upper right). * $p < 0.05$ compared with control, ** $p < 0.01$ compared with control, *** $p < 0.001$ compared with control; # $p < 0.05$ compared with 1 week, ### $p < 0.001$ compared with 1 week; & $p < 0.05$ compared with 1 month, & $p < 0.01$ compared with 1 month. N.S., No significant difference.

FIG. 3. Induction of hepatic gene expression, including hPXR target genes. (A) Hepatic expression of PXR target genes after rifaximin treatment was analyzed by quantitative real-time PCR analysis of mRNA from genes including hPXR, mouse *Cyp3a11*, *Gsta1*, *Ugt1a6*, *Mdr1a*, and *Oatp2*. As indicated, there is no induction of hepatic genes shown after 1 week treatment with rifaximin. However, long-term treatment with rifaximin (1M, 3M, and 6M) results in significant increase in the expression of hepatic PXR target genes (B) related to fatty acid transport (*Cd36*), triglyceride synthesis and transport (*Dgat2*), lipid storage (*Fsp27*, *Cfd*), and the nuclear receptors related to lipid metabolism (*Pparγ*, *Pparγ2*). These genes show increasing induction with time dependence of rifaximin treatment in hPXR mice. L, liver. * $p < 0.05$ compared with control, ** $p < 0.01$ compared with control, *** $p < 0.001$ compared with control; # $p < 0.05$ compared with 1 week, ## $p < 0.01$ compared with 1 week, ### $p < 0.001$ compared with 1 week; & $p < 0.05$ compared with 1 month, && $p < 0.01$ compared with 1 month, &&& $p < 0.001$ compared with 1 month; \$ $p < 0.05$ compared with 3 month, \$\$ $p < 0.01$ compared with 3 month, \$\$\$ $p < 0.001$ compared with 3 month.

PXR-dependent enhancement of rifaximin toxicity upon ligand activation.

Activation of Hepatic Human PXR and Gene Expression Related to Hepatic Lipid Metabolism by Rifaximin Administration

To determine the mechanism of the elevated hepatic triglycerides found after 6-month treatment with rifaximin, expression of PXR target genes after rifaximin treatment was analyzed by quantitative real-time PCR analysis of mRNA encoded by genes including hPXR, mouse *Cyp3a11* (cytochrome P450 3a11), *Gsta1* (glutathione S-transferase alpha 1), *Ugt1a6* (UDP-glucuronosyltransferase 1a6), *Mdr1a* (multidrug resistance 1a), and *Oatp2* (organic anion transporting polypeptide 2). *Cyp3a11*, *Gsta1*, *Ugt1a6*, *Mdr1a*, and *Oatp2* are regulated by PXR (Cheng *et al.*, 2011). Rifaximin was previously reported to only activate PXR target genes in the intestine and not liver after short-term

treatment of hPXR mice (Ma *et al.*, 2007). Indeed, no induction of hepatic genes was found after 1-week treatment with rifaximin, while long-term treatment (1, 3, and 6 months) significantly increased several hepatic PXR target genes (Fig. 3A) in hPXR mice; induction was not shown consistently in WT and *Pxr*-null mice (Supplementary fig. 3A).

Fatty liver is due to the altered expression of genes related to lipid metabolism (Brookheart *et al.*, 2009). The genes related to fatty acid transport: *Cd36*, *Fabp1* (fatty acid-binding protein 1), and *Ap2* (adipocyte protein 2); mitochondrial β -oxidation: *Cpt1a* (cholinephosphotransferase 1 alpha), *Acadm* (acyl-coenzyme A dehydrogenase for medium-chain), *Acadl* (acyl-CoA dehydrogenase for long-chain), *Acads* (acyl-CoA dehydrogenase for short-chain), *Acs1* (acyl-CoA synthetase long-chain), and *Acox1* (acyl-CoA oxidase 1); fatty acid synthesis: *Fasn* (fatty acid synthase), *Acc1* (acetyl-coA carboxylase 1), and *Scd1* (stearoyl-CoA desaturase 1); triglyceride synthesis and transport: *Dgat1*

(diacylglycerol O-acyltransferase 1), *Dgat2* (diacylglycerol O-acyltransferase 2), and *Mttp* (microsomal triglyceride transfer protein); lipid storage: *Fsp27* (fat-specific protein of 27kDa), *Cfd* (complement factor D (adipsin)); and the nuclear receptors related to lipid metabolism: *Srebp-1*, *Ppara*, *Ppar γ* , *Ppar γ 2*, and *Ppar δ* (peroxisome proliferator-activated receptor α , γ , γ 2 and δ). Among these genes, *Cd36*, *Dgat2*, *Ppar γ* , *Ppar γ 2*, *Fsp27*, and *Cfd* mRNAs were increased in hPXR mice (Fig. 3B), although not in WT and *Pxr*-null mice (Supplementary fig. 3A) after rifaximin treatment, when compared with only slight increases in the expression of mRNAs encoded by other genes related to lipid metabolism (Supplementary fig. 4).

Activation of Human PXR in Small Intestine and Gene Expression Related to Lipid Absorption by Rifaximin Administration

Lipid absorption in small intestine is the key step for lipid accumulation in the body. *Cd36*, intestinal-specific *Fabp2*, *ApoB* (apolipoprotein B), and *ApoA-IV* (apolipoprotein A-IV) are largely responsible for the uptake of triglycerides from the intestinal lumen to the enterocyte (Sandoval *et al.*, 2010). It was reported that PXR, PPARs, and LXRs (liver X receptor) can activate *Cd36* (Zhou *et al.*, 2006). As rifaximin is a gut-specific human PXR agonist, expression of mRNAs from PXR target genes including *Cyp3a13*, *Cyp3a11*, *Gsta1*, and *Mdr1a* were determined in the small intestine of rifaximin-treated hPXR mice. *Cyp3a13* is the most responsive PXR target gene in the small intestine. mRNAs from all target genes were increased by 1 week of rifaximin treatment and further increased after longer treatment (Fig. 4A). However, there was no significant induction of these mRNAs in WT or *Pxr*-null mice (Supplementary fig. 3B). *Cd36*, *Fabp2*, and *ApoA-IV* mRNAs were also induced and remained elevated for up to 6 months of rifaximin treatment (Fig. 4B). *Fabp2* is an intestine-specific protein responsible for fatty acid transport. *Cd36* and *ApoA-IV* are distributed in small intestine as well as liver and other tissue. The induction of these genes indicates that rifaximin administration increases lipid absorption in enterocytes in a human PXR-dependent manner, which is not found in WT and *Pxr*-null mice (Supplementary fig. 3B).

Protein and IHC Analysis of Fabp2 Induction and Lipid Content Increase with Rifaximin Administration

To confirm that up-regulation of *Cd36* and *Fabp2* mRNAs were also reflected at the protein level, small intestinal epithelial cell were isolated, whole cell protein of jejunum was extracted, and Western blot analysis was performed indicating a gradual up-regulation of *Cd36* and *Fabp2* in hPXR treated mice from 1 to 3 months on rifaximin, with no increase found in *Pxr*-null mice compared with control even up to 5 months on the drug (Fig. 5A). Additionally, jejunum sections from hPXR mice treated with rifaximin were highly immunostained compared with control sections, suggesting that the monoclonal anti-human *Fabp2* had some, but very low, crossreactivity with mouse *Fabp2*. Overall, the jejunum from treated mice showed more immunostaining than jejunum from

hPXR control mice (Fig. 5B). The high degree of immunostaining indicates that intestinal specific *Fabp2* is induced significantly via hPXR activation by rifaximin, which might influence lipid transport, as *Fabp2* has an essential role in fatty acid binding.

The levels of triglycerides, nonesterified free fatty acids, and cholesterol in epithelial cells was investigated through lipid extraction and analyzed. Increased triglycerides in enterocytes were observed in rifaximin-treated hPXR mice when compared with WT and *Pxr*-null mice (Fig. 5C). However, there was no significant increase in nonesterified free fatty acid and total cholesterol in hPXR enterocytes as well as WT and *Pxr*-null, indicating that triglyceride is a major component induced through rifaximin-activated lipid absorption through small intestine into the liver circulation. These data supported the interpretation that triglyceride uptake was induced in enterocytes from the proximal intestine of hPXR treated mice as a direct consequence of *Fabp2* activation (Levy *et al.*, 2009).

Metabolomics Analysis of Urine, Serum, Feces, and Liver Homogenate

Almost 98% of rifaximin is excreted directly into feces. To measure the flux of rifaximin in mice, metabolomics of urine, serum, feces, and liver homogenate were analyzed. Results revealed no clear separation between urine metabolites control and treated groups from 1 week to 6 months in hPXR, WT, and *Pxr*-null mice. Serum metabolomics analyzed by PCA (principle component analysis) revealed no clear clustering of controls from rifaximin-treated groups in hPXR mice. However, liver homogenate analysis in the MS negative mode revealed a clear clustering between control and 1 week rifaximin-treated hPXR mice versus 3-month rifaximin-treated mice plus 6-month rifaximin-treated hPXR mice. The model fit (R^2 value) and prediction power (Q^2 value) of PCA model was 0.63 and 0.104, respectively, indicating good separation of the PCA model (Fig. 6A). The loading plot showed contribution ions I, II, and III to be AMP, ADP (adenosine diphosphate), and ATP (Fig. 6B). The major fragments of ATP/ADP/AMP had the highest contribution to the clustering. As ADP and ATP are easily fragmented due to collision under MS analysis, a biochemical kit was used to identify the ADP and ATP levels in livers from rifaximin-treated hPXR mice, and AMP was quantified using a Xevo G2 QTOF. The results revealed that AMP:ATP value was significantly up-regulated in 6-month-treated hPXR mice (Fig. 6C). Furthermore, feces metabolomics in negative mode demonstrated clustering metabolites in hPXR mice with rifaximin treatment for 3 or 6 months, differentiated from control and 1-week treatment, as well as from WT control group, 6-month-treated group, *Pxr*-null control group, and 6-month-treated group (Fig. 7A). The R^2 value and Q^2 value of the PCA model were 0.781 and 0.371, respectively, which indicate a good model. Loading plots (Fig. 7B) demonstrated that multiple sulfonated bile acids contributed to the separation, and among them the most contributive ions were MSMS of 487.239⁻ (I) and 471.243⁻ (II). After *in vitro* incubation with

FIG. 4. hPXR target genes in small intestine show induction from 1-week treatment with rifaximin, which indicate a sensitive response of hPXR in small intestine to rifaximin. With the extension of treatment, the target genes are induced gradually (A). In addition, genes encoding Cd36, Fabp2, ApoA-IV, etc., in small intestine of hPXR mice treated with control or rifaximin for 1 week, 1 month, 3 months, and 6 months revealed PXR-dependent induction (B). I, small intestine. * $p < 0.05$ compared with control, ** $p < 0.01$ compared with control, *** $p < 0.001$ compared with control; # $p < 0.05$ compared with 1 week, ## $p < 0.01$ compared with 1 week, ### $p < 0.001$ compared with 1 week; \$ $p < 0.05$ compared with 3 month.

FIG. 5. Scrapped small intestinal epithelial cell were isolated. Results of Western blotting indicated a strong gradual up-regulation of Cd36 and Fabp2 in hPXR treated mice from 1 to 3 months, while not in *Pxr*-null mice compared control with 5 months of rifaximin treatment (A). Additionally, jejunum sections from hPXR mice treated with rifaximin were highly immunostained compared with control sections, suggesting that the monoclonal anti-human Fabp2 had some, but very low, cross-reactivity with mouse Fabp2. Overall, the jejunum from treated mice showed more immunostaining than jejunum from hPXR control mice (B). Pooled whole cell extract samples ($n \geq 6$ small intestine in each group, 40 μ g total loading proteins) were used for Western blot analysis. Anti-rabbit polyclonal antibody against Cd36 and anti-goat polyclonal antibody against Fabp2 specifically recognizes these two proteins. β -actin was used as a loading control. Anti-goat antibody against Fabp2 was diluted as 1:1000 and used on immunohistochemistry of Fabp2 in jejunum epithelial cells. Intestinal villi with rifaximin treatment showed strong staining compared with control. Magnification: $\times 200$. Moreover, high affinity saturable component of uptake was increased in enterocytes from the proximal intestine of rifaximin-treated hPXR mice as a direct consequence of Fabp2 activation. An increase in triglyceride (TG) in enterocytes and no increase in fatty acid (NEFA) and cholesterol (CHOL) was observed, compared with the levels in hPXR compared to WT as well as no change between WT and *Pxr*-null mice (C). *** $p < 0.001$ compared with WT.

FIG. 6. Liver homogenate metabolomics in negative mode revealed a clear clustering between control (●) plus 1-week treated hPXR mice (■) versus 3-month treated mice (▲) plus 6-month treated hPXR mice (◆). The model fit (R^2 value) and prediction power (Q^2 value) of the PCA model was 0.63 and 0.104, respectively, which indicate good separation of PCA model (A). Loading plot showed contribution ions I, II, and III to be AMP, ADP, and ATP (B). As ADP and ATP are easily fragmented due to collision under mass spectrum analysis, the biochemical kit is used to identify the ADP and ATP levels in liver of mice treated with rifaximin in hPXR mice, and AMP was quantified by using Xevo G2 QTOF. Results revealed that the AMP:ATP value was significantly up-regulated in 6-month treated hPXR mice (C). * $p < 0.05$ compared with control; # $p < 0.05$ compared with 1 week; & $p < 0.05$ compared with 1 month; \$ $p < 0.05$ compared with 3 month.

FIG. 7. Feces metabolomics in negative mode demonstrated the clustering in hPXR mice with rifaximin treatment for long-term 3- (▲) or 6- (▼) month differentiated from control (●) and 1-week (■) treatment, as well as from WT control group (●), 6-month treated group (▼) and *Pxr*-null control group (●) and 6-month treated group (▼) (A). The R^2 value and Q^2 value of the PCA model were 0.781 and 0.371, respectively, which indicate a good model of PCA. Loading plots (B) demonstrated that multiple sulfonated bile acids contributed to the clarification, among them, the most contributive ions were MSMS of 487.239⁻ (I) and 471.243⁻ (II). After *in vitro* incubation with sulfatase, these two target ions were digested into 407.238⁻ and 391.243⁻ with deletion of a sulfate base (MW = 80). The markers were identified to be mixture of ursodeoxycholic acid (UDCA) and deoxycholic acid (DCA) (407.238⁻) (C) as well as a mixture of tauro- α -muricholic acid (T- α -MCA), tauro- β -muricholic acid (T- β -MCA), and cholic acid (CA) (391.237⁻) (D), as demonstrated by tandem MS and comparison with authentic standards.

sulfatase, these two target ions were converted to 407.238⁻ and 391.243⁻ with loss of the sulfate base (MW = 80). The digestive markers were identified to be a mixture of ursodeoxycholic acid (UDCA) and deoxycholic acid (DCA; 407.238⁻) (Fig. 7C) as well as a mixture of tauro- α -muricholic acid (T- α -MCA), tauro- β -muricholic acid (T- β -MCA), and cholic acid (CA; 391.237⁻) (Fig. 7D), as demonstrated by tandem MS and comparison with authentic standards. The expression of hepatic Cyp7a1 (cholesterol 7 α -hydroxylase), Cyp7b1 (25-hydroxycholesterol 7 α -hydroxylase), and FXR (farnesoid X receptor) were significantly induced only in hPXR mice (Supplementary fig. 5), which indicated increased synthesis of bile acids in the liver after rifaximin. In contrast, mRNA of genes encoding Cyp27a1 (sterol 27-hydroxylase), Bsep (bile salt export pump), Oatp1, Oatp2, and Oatp4 (organic anion transporting polypeptide 1, 2, 4), which are responsible for bile acid transport, were not significantly changed by rifaximin treatment. Therefore, the high distribution of bile acids and bile acid conjugates in feces of hPXR treated with rifaximin reveals an accumulation of bile acid that might be due to triglyceride overload in the circulation leading to increased synthesis of bile acid (Brunet *et al.*, 1999; Smelt, 2010) and overexcretion of bile acid in the

feces, while this up-regulation was not revealed in WT as well as *Pxr*-null mice.

DISCUSSION

Rifaximin is used as a therapy for gastrointestinal diseases such as traveler diarrhea and hepatic encephalopathy, with recent clinical trials inciting its efficacy in IBS. However, as the agonist of PXR, the effects of long-term induction of PXR on intestinal and liver physiology and drug metabolism merits further investigation. PXR was recently reported to be an important endobiotic nuclear receptor regulating endogenous homeostasis, especially influencing steroids, bile acids, glucose, energy metabolism, and inflammation (Cheng *et al.*, 2012). It was reported that human PXR gene variants are associated with liver injury in nonalcoholic fatty liver disease (NAFLD) where PXR polymorphisms enhance the susceptibility to progression to more severe stages of NAFLD (Sookoian *et al.*, 2010). Others investigated 76 different nuclear receptor ligands and their effect on NAFLD by evaluation of fat content in human hepatocytes and hepatoma cells after ligand treatment (Moya *et al.*, 2010). The results revealed that overexpression of

PXR in HepG2 cells enhanced the steatogenic effect of hyperforin and rifampicin. In contrast, accumulation of fat induced by other ligands did not correlate with the expression of their associated nuclear receptor. Moreover, the mouse PXR ligand PCN induced a decrease in plasma LDL-cholesterol levels and increase in triglyceride-rich VLDL particles (Hoekstra *et al.*, 2009). In the liver, PCN induced a significant increase in the level of triglycerides and phospholipids, which might be through activation of SREBP (Zhou and Xie, 2006) and other transcription factors (Moreau *et al.*, 2009; Roth *et al.*, 2008).

Different from other PXR ligands, rifaximin is a gut-specific human PXR ligand that is not significantly absorbed into circulation. Metabolomic analysis revealed no detectable rifaximin in the liver, serum, and urine. The current study established that long-term treatment with rifaximin causes hepatic triglyceride accumulation that might be due in part to the up-regulation of triglyceride uptake and synthesis in the intestine. Secreted triglycerides were increased in hPXR mouse intestine but not in WT and *Pxr*-null mice, and this was correlated with induction of intestinal genes responsible for fatty acid uptake (*Cd36*, *Fabp2*), triglycerides synthesis (*Dgat1* and *Dgat2*), as well as the formation of LDL-cholesterol (*ApoB*) and chylomicrons (*ApoA-IV*; Green and Riley, 1981). *Cd36* is the transporter for fatty acid uptake in enterocytes and liver (Martin *et al.*, 2011), whereas *Fabp2* is the intestinal fatty acid binding protein responsible for intestinal-specific activation of lipid metabolism or transport (Cianflone *et al.*, 2008) through gut-specific human PXR activation. Triglycerides are absorbed and rebuilt in the enterocytes and packaged with cholesterol and proteins to form chylomicrons, which are excreted into the blood and then triglycerides are stored in hepatocytes as a source of energy (Mellitzer and Gradwohl, 2011). Subsequently, as endogenous agonists for multiple nuclear receptors, lipid metabolites can induce hepatic PXR (Sui *et al.*, 2011), PPAR α and γ , (Kisfali *et al.*, 2010), FXR (Yang *et al.*, 2010), and other receptors responsible for lipid transport and metabolism.

Increased bile acid levels were also found in feces from rifaximin-treated hPXR mice and not in WT and *Pxr*-null mice, which might reflect the influence of triglycerides on bile acid synthesis, transport, and excretion in the enterohepatic circuit. In particular, the major ions of T- α , β -MCA, CA and UDCA, and DCA, which are important primary bile acids for facilitation of fat absorption and cholesterol excretion, are increased in the rifaximin-treated hPXR mice. Hepatic gene expression analysis showed that *Cyp7a1*, *Cyp7b1*, as well as FXR mRNAs were significantly induced only in rifaximin-treated hPXR mice. *Cyp7a1* is the rate-limiting enzyme in the synthesis of bile acids from cholesterol, catalyzing the formation of 7 α -hydroxycholesterol (Lorbek *et al.*, 2012). CYP7A1 is down-regulated by SREBP when plasma cholesterol levels are low (Ponugoti *et al.*, 2007). The increase in bile acids upon long-term rifaximin exposure might be through increased production of bile acids and reduced levels of cholesterol in hepatocytes. Increased bile acid and triglycerides regulate the target genes such as FXR and PXR and

then the downstream genes are subsequently controlled in liver. Specifically, an increase in the AMP *versus* ATP ratios in livers of rifaximin-treated hPXR mice were noted in the hPXR mice exposed to rifaximin for 6 months compared with the control group. The AMP/ATP ratio is a biomarker for fatty liver progression (Viollet *et al.*, 2009). It was reported that an increase of the AMP/ATP ratio causes activation of AMP-activated protein kinase (AMPK; Hardie *et al.*, 2012) in the liver leading to the stimulation of fatty acid oxidation and inhibition of lipogenesis, glucose production, and protein synthesis. Clinical studies revealed an increase of AMPK activity in patients with hepatic metabolism disorders associated with induction of AMP *versus* ATP ratios. The present results indicate that rifaximin-induced fatty liver has induction of AMPK activity through observation of metabolomics change of AMP and ATP levels in liver.

It should be mentioned that although the potential toxicity of rifaximin is observed after a 6-month treatment regimen with rifaximin, the lifespan of mice *versus* human is equivalent to 1:50. Thus, the 6-month treatment with rifaximin corresponds to a 12-year treatment of rifaximin in humans, and that lower toxicity occurring from 1-month treatment with rifaximin to mice roughly equals four years of treatment in humans. Clinical therapy of rifaximin for traveler's diarrhea takes 3–6 days (Taylor *et al.*, 2006), and a 10-day regimen is used to treat small bowel bacterial overgrowth (Corazza *et al.*, 1992) or pseudomembranous colitis (Nelson, 2007). Over half a year is used to treat hepatic encephalopathy (Macclayton and Eaton-Maxwell, 2009), and 10 days to half a year was used to perform the phase 3 clinical trial to treat irritable bowel syndrome (Pimentel, 2009). There were no significant adverse events reported during the clinical trials of rifaximin performed to date. Even in more vulnerable patient populations, such as in patients with chronic liver disease treated with rifaximin at the 550mg b.i.d, a comparable incidence of minor adverse events was noted between rifaximin and placebo (Kalambokis and Tsianos, 2012). Nevertheless, there remains a concern that, at least theoretically, treatment with rifaximin would lead to an increased incidence of resistant enteric microorganisms. Isolated reports have described the emergence of resistant Staphylococcal species in the skin of healthy volunteers associated with rifaximin administration (Valentin *et al.*, 2011) and the existence of resistant *Clostridium. difficile* strains *in vitro* (Huhulescu *et al.*, 2011).

In summary, this is the first study in a humanized mouse model to investigate the long-term function of rifaximin's effect on physiology through PXR activation. Hepatocellular fatty degeneration emerges with a time-dependent manner, with increased expression of intestinal lipid uptake transporters (*Cd36* and *Fabp2*) as well as the up-regulation of triglyceride secretion in enterocytes, associated with augmented metabolism of ATP and overexcretion of bile acid into feces in hPXR mice as summarized in Figure 8. Most changes observed were human PXR-dependent and not seen in similarly treated WT and *Pxr*-null mice, thus indicating that rifaximin induces hepatocellular fatty degeneration through human PXR activation. However, despite the potential adverse effect of long-term

FIG. 8. Rifaximin-induced hepatotoxicity via human PXR activation. Major metabolic pathways encompassing rifaximin induction of hPXR and lipid transportation genes (Cd36, Fabp2, Dgat1, and Dgat2 etc.) in small intestine, then triglyceride overload in liver, which lead to reduced cholesterol and increased bile acids. hPXR target genes and lipid metabolism genes are increased in liver, due in part to bile acid and triglycerides accumulation, and subsequently up-regulated hepatic nucleotide phosphates and bile acid abundance in feces. TG, triglycerides.

administration of rifaximin, it is still within a safety tolerance applied in clinical therapy to treat gastrointestinal diseases.

SUPPLEMENTARY DATA

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

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