FXR activation by obeticholic acid or nonsteroidal agonists induces a human-like lipoprotein cholesterol change in mice with humanized chimeric liver

Romeo Papazyan, * Xueqing Liu, * Jingwen Liu, † Bin Dong, † Emily M. Plummer, * Ronald D. Lewis II, * Jonathan D. Roth, * and Mark A. Young †

Intercept Pharmaceuticals, Inc., * San Diego, CA 92121; and Veterans Affairs Palo Alto Health Care System, † Palo Alto, CA 94304

Abstract Obeticholic acid (OCA) is a selective farnesoid X receptor (FXR) agonist that regulates bile acid and lipid metabolism. FXR activation induces distinct changes in circulating cholesterol among animal models and humans. The mechanistic basis of these effects has been elusive because of difficulties in studying lipoprotein homeostasis in mice, which predominantly package circulating cholesterol in HDLs. Here, we tested the effects of OCA in chimeric mice whose livers are mostly composed (>80%) of human hepatocytes. Chimeric mice exhibited a human-like ratio of serum LDL cholesterol (LDL-C) to HDL cholesterol (HDL-C) at baseline. OCA treatment in chimeric mice increased circulating LDL-C and decreased circulating HDL-C levels, demonstrating that these mice closely model the cholesterol effects of FXR activation in humans. Mechanistically, OCA treatment increased hepatic cholesterol in chimeric mice but not in control mice. This increase correlated with decreased SREBP-2 activity and target gene expression, including a significant reduction in LDL receptor protein. Cotreatment with atorvastatin reduced total cholesterol, rescued LDL receptor protein levels, and normalized serum LDL-C. Treatment with two clinically relevant nonsteroidal FXR agonists elicited similar lipoprotein and hepatic changes in chimeric mice, suggesting that the increase in circulating LDL-C is a class effect of FXR activation. —Papazyan, R. X. Liu, J. Liu, B. Dong, E. M. Plummer, R. D. Lewis II, J. D. Roth, and M. A. Young. FXR activation by obeticholic acid or nonsteroidal agonists induces a human-like lipoprotein cholesterol change in mice with humanized chimeric liver. J. Lipid Res. 2018. 59:982–993.

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The farnesoid X receptor (FXR) is a ligand-activated nuclear receptor/transcription factor that regulates bile acid homeostasis, lipid and glucose metabolism, and hepatic inflammation and fibrosis (1,2). FXR knockout mice present with elevated cholesterol and triglycerides in serum and liver (3,4). These mice are prone to an atherogenic phenotype (5), mediated by the collective contribution of elevated circulating non-HDL cholesterol (non-HDL-C) and triglyceride levels, increased apolipoprotein B-containing lipoprotein synthesis, and increased intestinal cholesterol absorption (5). By contrast, in mice with intact FXR signaling, FXR ligands target lipid pathways to reduce hepatic and circulating triglycerides by repressing SREBP1c and its lipogenic targets (6), increasing expression of ApoCII and phospholipid transfer protein (7) and maintaining elevated levels of VLDL receptor (8). FXR targets also reduce circulating cholesterol by induction of scavenger receptor B1 (SCARB1/SR-B1) and ATP-binding cassette G8 transporter (4,9), proteins important in reverse cholesterol transport. Excretion of cholesterol may also occur across the intestine through a pathway dependent on intestinal FXR (10). Together, these results suggest that activation of FXR exerts significant hypolipidemic effects in both the liver and circulation (3). These hypolipidemic and antiatherogenic effects of FXR activation have been demonstrated across studies in a variety of animal models (2,11–14).

With the recognition that FXR has pleiotropic actions in regulation of bile acid and lipid metabolism, glucose regulation, and inflammation, FXR agonists are a current focus of therapeutic development in metabolic and inflammatory disease. Obeticholic acid (6-ECDCA; INT-747;...
OCA; OCALIVA™) is a bile acid analog and selective FXR agonist with limited to no activity on TGR5 (15). OCA is indicated for the treatment of primary biliary cholangitis in combination with ursodeoxycholic acid (UDCA) in adults with an inadequate response to UDCA or as monotherapy in adults unable to tolerate UDCA (16). OCA is also in late-stage development for nonalcoholic steatohepatitis (NASH) (17) and early stage development for other chronic liver diseases. The primary action of OCA in cholesterol is the reduction in bile acid synthesis by repression of the cytochrome P450 gene encoding cholesterol 7α-hydroxylase (CYP7A1) expression and elevated expression of bile acid transporters (15) to reduce bile acid toxicity and inflammation (18). In rabbit (19, 20) and rodent (21, 22) models of fatty liver disease, OCA reduces circulating cholesterol and is involved in pathways contributing to the resolution of steatosis, inflammation, and fibrosis. In humans, similar hepatic histological improvements are evident; however, the impact on circulating lipoprotein profiles differs from that which is observed in preclinical models (17). For example, in healthy volunteers, OCA induced a sustained elevation of serum LDL cholesterol (LDL-C) concentration (+22%) and reduction of HDL-C (~16%), with a small elevation of total cholesterol (23). A similar profile was noted in individuals with type 2 diabetes mellitus (24) and in patients with biopsy-confirmed NASH (17). In the context of primary biliary cholangitis, the rise in LDL-C was smaller and transient, but was still accompanied by a decrease in HDL-C (16). Human studies using the endogenous bile acid FXR agonist chenodeoxycholic acid (CDCA) have reported elevated LDL-C (25–27) similar to that reported with OCA, with no observed changes in serum HDL-C. To date, animal studies demonstrating FXR-mediated reductions across lipoprotein cholesterol fractions (1, 11, 13, 21, 28–32) do not adequately predict the changes observed in human clinical trials.

These discrepancies demonstrate that signaling pathways regulating lipoprotein cholesterol metabolism by FXR activation are incompletely understood. The similarity in LDL-C reduction observed in humans and animals suggests underlying mechanisms common to animals and humans, postulated to result in part from induction of SR-B1 (4, 9). However, the effects of FXR activation on LDL-C vary in different assay systems. FXR activation by CDCA or the nonsteroidal agonist GW-4064 increased LDL receptor (LDLR) mRNA in human hepatocyte cell culture (33–35), accompanied by increased binding activity of LDLR. These actions of FXR potentiated the effect of pravastatin by counteracting the statin-induced increase in proprotein convertase subtilisin/kexin type 9 (PCSK9) expression, resulting in increased LDLR activity (33). These in vitro responses are consistent with an LDL-lowering effect of FXR. However, they contrast with clinical observations where CDCA administration for 3 weeks to healthy volunteers and surgical patients reduced mRNA expression of LDLR in liver biopsy (26), reduced LDL-C fractional clearance (27), and modestly increased circulating LDL-C (26, 27). In other words, with respect to circulating lipoproteins, preclinical animal models and in vitro human systems fail to faithfully recapitulate the clinical experience.

Chimeric mice with transplanted human hepatocytes exhibit a “humanized” circulating lipoprotein cholesterol profile with an LDL-C/HDL-C ratio similar to that observed in humans, as well as bile acid regulation more characteristic of humans (36, 37). The primary aim of the current study was to determine whether mice with humanized chimeric livers could reproduce the OCA-associated response in circulating lipoproteins observed in clinical trials. Second, we sought to determine whether the effects of OCA could be attenuated by administration of statin therapy. Lastly, given the potential for bile acid-derived FXR agonists to have actions different from nonsteroidal agonists (38, 39), we compared the actions of OCA with two nonsteroidal FXR agonists currently being evaluated in clinical trials (40–44). Our results indicate that the humanized mouse exhibits FXR-mediated lipoprotein changes similar to those reported in humans, and we put forth a mechanism that distinguishes the FXR response in humanized mice from that in control mice.

MATERIALS AND METHODS

Animals

Chimeric liver mice (cDNA-uPA<sup>野生/+/SCID</sup>) were generated by PhoenixBio Co. Ltd. (Higashi-Hiroshima, Japan) by the methods described previously (45, 46). PhoenixBio Japan provided and performed studies on PXB mice and SCID mice (nonchimeric; same SCID background as PXB mice). The replacement index of human hepatocytes as determined by human albumin levels in serum from PXB mice was ~80% in mice used throughout the study. All mice were male and 12–18 weeks old on day 1. Mice were fed a chow diet [CRF-1 (supplemented with 300 mg of vitamin C per 100 g of chow); Oriental Yeast Co. Ltd., Japan] and were fasted for 4 h prior to euthanizing. All procedures involving animals were performed humanely. All animal experiments have been approved by the Animal Ethics Committee of PhoenixBio.

Synthesis of tool compounds

The tool compounds studied include 6a-ethyl-3α,7α-dihydroxy-5β-cholan-24-oic acid (OCA; INT-747), 2-[3-((5-cyclopropyl-3-[2-( trifluoromethoxy)phenyl]1,2-oxazol-4-yl)methoxy)-8-azabicyclo [3.2.1] octan-8-yl)4fluoro,3-benzothiazole-6-carboxylic acid (1-lB; INT-2228), and 2-3-(2-chloro-4-(3-cyclopropyl-3,2,6-dichloro-phenyl)isoxazol-4-yl)methoxy)phenyl)-3-hydroxazetidin-1-yl) isonicotinic acid (13/9; INT-2231) (see Fig. 5A). The synthesis of OCA can be performed following previously reported procedures by Pellicciari et al. (18). Nonsteroidal tool compounds INT-2228 and INT-2231 were prepared following detailed published procedures by Tully et al. (43, 44) and Kinzel et al. (42). Analytical data for the synthesized tool compounds conformed with previously reported analytical information.

Dosing

Mice were dosed orally (p.o.) once per day (QD) for 14 days with vehicle (0.5% carboxymethyl cellulose) or 10 mg/kg/day OCA, 10 mg/kg/day atorvastatin, or both in combination. In the follow-up study with nonsteroidal agonists, PXB mice were dosed p.o. QD for 14 days with vehicle (1% carboxymethyl cellulose),
OCA (10 mg/kg/day), or INT-2228 (0.3 mg/kg/day) and twice per day (BID) for 14 days with INT-2231 (30 mg/kg BID). The final dose for each mouse was administered the day before their euthanization. Control mice (SCID) were not studied with INT-2228 or INT-2231.

Blood, tissue preparation, and metabolite measurements

On either day −4 or day 1 (predose) after 4 h of fasting, mice were anesthetized with isoflurane, and blood was collected via retro-orbital sinus for baseline measurements. On day 15, mice were fasted for 4 h, anesthetized for blood collection, and euthanized by cardiac puncture and exsanguination. For all timepoints, serum was separated by centrifugation.

At termination, livers were removed, and portions were reserved in RNA Later (ThermoFisher Scientific) for quantitative PCR (qPCR) and frozen separately for lipid analysis and Western blotting. Serum cholesterol was measured by Skylight Biotech Inc. (Akita, Japan) using the LipoSEARCH gel permeation HPLC platform (47). Total serum cholesterol was calculated by adding concentrations (mg/dl) of chylomicron, HDL, LDL, and VLDL.

Hepatic lipids were extracted at Skylight Biotech according to the Folch method (48). Briefly, liver pieces (approximately 30 mg) were homogenized in chloroform/methanol (2/1, vol/vol) solution. The organic phase was dried and resolubilized in 2-propanol. Total liver cholesterol and triglycerides (Sekisui Medical Co., Tokyo, Japan) as well as serum enzymes (Fujifilm Corp., Tokyo, Japan) were measured by Skylight Biotech using commercial kits.

Western blotting, qPCR analysis, and ELISAs

Liver samples from PXB and SCID mice (vehicle-treated, OCA-treated, atorvastatin-treated, or OCA+atorvastatin-treated) were preserved in RNA Later (ThermoFisher Scientific), and total RNA was isolated. Reverse transcription was performed by using the RT² First Strand Kit (Qiagen, Manchester, UK; catalog no. 330401), according to standard protocol. Changes in the expression of human and mouse genes involved in cholesterol metabolism (see Figs. 1, 3, and 4 and supplemental Fig. 5) were analyzed by using Custom Human RT² Profiler PCR Array and Custom Mouse RT² Profiler PCR Array (SA Biosciences, Qiagen), which contained proprietary primer sequences. The assay was performed on a Bio-Rad CFX Connect Real-Time PCR Detection System. The relative expression (fold change) was calculated by dividing normalized mRNA levels of the test sample with the normalized mRNA levels of the vehicle-treated sample. The reference gene used for normalization in human samples was RPLP0 and in mouse samples was Gusb.

In the follow-up study, total RNA was extracted from frozen liver samples from PXB mice (vehicle-treated, OCA-treated, INT-2228-treated, or INT-2231-treated) by using RNA Lysis Buffer and purified by using the Zymo-Spin Columns (Quick-RNA™ Mini-Prep Kit from Zymo Research, catalog no. R1054). For the data in Fig. 3, qPCR was performed as previously described (21) and normalized to the reference human gene RPLP0. Primer sequences for these experiments are listed in supplemental Table 3.

SDS-PAGE and Western blotting were performed as previously reported (21). Antibodies used for Western blotting included rabbit anti-LDLR antibody (Biovision, Mountain View, CA; catalog no. 3839), rabbit anti-SREBP2 antibody [gift from Dr. Sahng Wook Park (Yonsei University, Seoul, South Korea)], and monoclonal anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO; catalog no. A1978). All primary antibodies were used at 1:1,000 dilution, and the secondary antibody dilution was 1:10,000. Immunoreactive bands of predicted molecular mass were visualized by using SuperSignal West Substrate (Thermo Scientific) and quantified with the Alpha View Software. Western/SDS-PAGE gels were parallel-processed in duplicate, and Western band intensities were first normalized to pooled samples in each respective membrane and then to the βACTIN signal of one of the membranes.

PXB mouse serum PCSK9 was directly measured by using the human PCSK9 Quantikine ELISA kit (R&D Systems; catalog no. DPC900). ELISAs for serum apolipoproteins were performed at Skylight Biotech by using commercial kits: APOA1, apoa-I AUTO.N “Daichi” kit (Sekisui Medical Co.); and APOB100 Human ApoB-100 Assay kit (catalog no. 27181, Immuno-Biological Laboratories Co., Ltd.)

Statistics

MS Excel and GraphPad Prism 7 were used to calculate averages and errors, generate graphs, and perform statistical tests. Unless otherwise indicated, a two-tailed and unpaired Student’s t test was used to compare two groups, and the ANOVA model was used to compare groups of three or more. Corrections used for multiple comparisons are indicated in the figure legends. Graphs represent mean ± SEM unless otherwise indicated. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001. A value of <0.05 was considered significant.

RESULTS

PXB mice exhibit a human-like lipoprotein cholesterol profile

Chimeric mice with humanized livers have shown promise in modeling human-specific drug responses previously unattainable in preclinical research (49). In the chimeric FRG (Fah−/−, Rag2−/−, Il2rg−/−) mouse model (36), lean mice exhibited a human-like ratio of serum LDL-C/HDL-C, suggesting that chimeric mice mitigate some key differences in cholesterol metabolism between humans and mice. In this study, the serum lipoprotein profile of another widely used chimeric model, PXB mice (produced by PhoenixBio Co., Ltd.), was characterized.

To highlight species-specific cholesterol effects, serum cholesterol levels in humanized PXB mice (cDNA-uPAwild/+/SCID) were compared with those of control (SCID) mice. The replacement index of human hepatocytes predicted by measurement of human albumin was ≥80% in all PXB mice examined. Whereas most of the serum cholesterol was expectedly packaged in HDL particles in control mice, PXB mice had enriched levels of LDL-C at baseline (Fig. 1A, B). The LDL-C/HDL-C ratio was 2.40 in PXB mice (Fig. 1C), which is similar to the ratio reported in humans (23, 37). Within LDL-C species, there was an enrichment of medium to large particles (fractions 8–9; Fig. 1B). The ratio of VLDL-C to total cholesterol was also significantly elevated (Fig. 1D). Thus, PXB mice exhibit a human-like lipoprotein profile at baseline.

To assess whether PXB mice appropriately model human hepatocyte-intrinsic responses to FXR activation, hepatic gene expression analysis was performed in animals treated with OCA (10 mg/kg/day). Previous studies have shown that FXR agonists increase fibroblast growth factor 19 (FGF19) mRNA expression in human hepatocytes, but not in mouse hepatocytes (murine homolog is Fgf15) (50–52). Consistent with these observations, OCA treatment induced
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hepatic FGF19 mRNA expression in PXB mice, whereas Fgf15 mRNA levels in control livers and FGF19 levels in untreated PXB livers remained virtually undetectable (Ct-values around 38) (Fig. 1E). These results demonstrate that human-specific molecular responses to OCA administration are intact in PXB mouse livers.

OCA treatment in PXB mice increases serum LDL-C that is attenuated by atorvastatin coadministration

Having established that PXB mice exhibit a human-like LDL-C/HDL-C ratio, we next tested whether chimeric mice accurately reproduce the effects of FXR activation seen in clinical trials. PXB and control (SCID) mice were treated for 2 weeks with OCA (10 mg/kg/day) and were compared with vehicle-treated mice. Body weights remained stable throughout the study, and measurement of serum alanine aminotransferase/aspartate aminotransferase indicated that OCA administration was not hepatotoxic in PXB mice (supplemental Fig. 1A, B). While there were no differences in serum cholesterol among the groups at baseline (supplemental Table 1), OCA treatment in control mice decreased levels of total serum cholesterol (Fig. 2A). This decrease manifested almost entirely from reduced levels of serum HDL-C, while no changes were detected in VLDL-C and LDL-C concentrations in control mice. In contrast, OCA treatment in PXB mice elicited significantly increased levels of LDL-C and decreased levels of HDL-C, which led to a net elevation in total serum cholesterol (Fig. 2A and supplemental Fig. 2). There were no OCA-induced changes to VLDL-C, suggesting that LDL-C

Fig. 1. PXB mice exhibit a human-like lipoprotein cholesterol profile. A, B: Representative serum lipoprotein measurements by gel permeation HPLC from control (SCID) and PXB mice. The x-axes are fractions where the indicated lipoproteins elute. CM, chylomicron. Data points represent mean ± SD of n = 9 mice per group. These mice have been treated with vehicle and are included in the analyses for Figs. 1–4. C: Serum LDL-C/HDL-C ratio calculated by dividing total LDL-C (fractions 8–13) with total HDL-C (fractions 14–20). Significance was determined by Student's t-test. n = 9 mice each group. D: Serum VLDL-C/total cholesterol ratio calculated by dividing total VLDL-C (fractions 3–7) with total cholesterol (fractions 1–20). Significance was determined by Student's t-test. n = 9 mice per group. E: Liver mouse Fgf15 and human FGF19 gene expression analysis by qPCR of control (SCID) and PXB mice, respectively. Control mice: n = 5 mice per group. PXB mice: Significance was determined by Student's t-test, n = 8 or 9 mice per group. * P < 0.05; **** P < 0.0001.
Fig. 2. OCA treatment in PXB mice increases serum LDL cholesterol that is attenuated by atorvastatin coadministration. A: Serum lipoprotein measurements from control (SCID) and PXB mice were determined by gel permeation HPLC. Shown are the cholesterol levels that changed from baseline in response to vehicle, OCA (10 mg/kg/day), and/or atorvastatin (ATV; 10 mg/kg/day) treatment. Control: Significance was determined by Student’s t-test. n = 9 mice per group. PXB: Significance was determined by one-way ANOVA with Tukey’s correction. n = 8 or 9 mice per group. B, C: Protein ELISA measurements (absolute values) of indicated lipoproteins from PXB mice. Significance was determined by one-way ANOVA with Tukey’s correction. n = 8 or 9 mice per group. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001; n.s., not significant.
and HDL-C metabolism are the main targets of OCA treatment in humanized mice. Consistent with our cholesterol measurements, ELISA analysis of serum lipoproteins in PXB mice revealed increases in LDL-associated APOB100 levels and decreases in HDL-associated APOA1 levels in response to OCA (Fig. 2B, C).

Because adjunctive statin coadministration reversed the OCA-induced increase in LDL-C levels in humans (53, 54), we tested whether this effect could be reproduced in PXB mice. Cotreatment with low-dose (10 mg/kg/day) atorvastatin prevented the increase in serum cholesterol levels associated with OCA administration (Fig. 2A). LDL-C levels were significantly reduced in cotreated mice compared with mice treated with OCA alone. Apolipoprotein levels determined by ELISA (Fig. 2B, C) supported the changes seen with direct cholesterol measurement. Together, these data indicate that PXB mice reproduce the effects associated with OCA treatment in humans and therefore can serve as a translational model to explore OCA-induced mechanisms.

**Liver cholesterol levels are increased in OCA-treated chimeric mice**

A major consequence of FXR activation is inhibition of bile acid synthesis and, by extension, decreased cholesterol excretion, but studies in rodent models have not produced an expected increase in liver cholesterol levels. Livers of PXB mice treated with OCA were harvested after a 2 week dosing regimen. While there were no major changes detected in control mice, OCA treatment in PXB mice led to a significant increase in total hepatic cholesterol (Fig. 3A). Atorvastatin, alone or in combination with OCA, reduced total intracellular cholesterol to levels below vehicle-treated animals (Fig. 3A). There were no changes in hepatic triglycerides or in lipogenic gene expression with OCA treatment (Fig. 3B, C), which suggests that VLDL synthesis was not altered after 2 weeks of treatment with a relatively mild OCA dose. Thus, attenuated bile acid synthesis and unaltered VLDL synthesis likely contributed to the increased levels of hepatic cholesterol in OCA-treated PXB mice. Moreover, given that VLDL-C particles are metabolized to LDL-C in circulation, these data demonstrate that OCA-dependent accumulation of serum LDL-C most likely results from decreased systemic clearance rather than increased VLDL secretion.

**Cholesterogenic gene expression is reduced by OCA and is normalized by atorvastatin coadministration in humanized mice**

To determine the status of hepatic cholesterol metabolism in OCA-treated chimeric mice, we next investigated levels of cholesterogenic gene expression. FXR target engagement was similar between control and PXB mice treated with OCA (Fig. 4A), with a modest repression of CYP7A1, and induction of small heterodimer partner (SHP/NR0B2), SCARB1 (SR-B1), and organic solute transporter (OST) β (SLC51B). As generally reported for FXR activation in rodents (21, 30), cholesterogenic genes were trending toward an increase with OCA treatment in control mice (Fig. 4B). These genes were significantly reduced in humanized PXB livers in response to OCA (Fig. 4C). Notably, cotreatment with atorvastatin normalized cholesterogenic gene expression in OCA-treated chimeric mice (Fig. 4C). The expression level of hepatic LDLR, a critical factor in circulating LDL-C clearance, was also reduced in OCA-treated PXB mice (Fig. 4C). Reduction in CYP7A1 mRNA expression correlated with LDLR mRNA levels in individual PXB mice (Fig. 4D), suggesting that LDLR levels were proportionally affected by the extent of FXR activation in each mouse. Serum LDL-C and hepatic LDLR mRNA expression were inversely correlated in individual mice, as expected (supplemental Fig. 3A).

SREBP-2 activation (by proteolytic cleavage) is critical for cholesterogenic gene expression in the liver and is regulated through several cholesterol-mediated feedback mechanisms (55). When intracellular cholesterol concentrations are low, the N terminus of precursor SREBP-2 (pSREBP-2) is cleaved to release a soluble, mature SREBP-2 fragment (mSREBP-2) that potently stimulates cholesterogenic genes. Mechanistically, the increased amounts of intracellular cholesterol and reduced cholesterogenic gene expression in OCA-treated PXB livers implied reduced

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**Fig. 3.** Liver cholesterol levels are increased in OCA-treated chimeric mice. A: Measurement of liver cholesterol levels in control (SCID) and PXB mice treated with OCA (10 mg/kg/day) and/or atorvastatin (ATV; 10 mg/kg/day). Total cholesterol was determined by an in vitro enzymatic assay. Control: Changes were not significant by Student’s ttest. n = 9 mice per group. PXB: Significance was determined by one-way ANOVA with Tukey’s correction. n = 8 or 9 mice per group. B: Measurement of liver triglycerides by in vitro enzymatic assay. Changes were not significant by Student’s ttest (Control) or one-way ANOVA (PXB). C: qPCR analysis of human lipogenic gene expression in PXB mouse livers. ***P < 0.001; ****P < 0.0001; n.s., not significant.
Cholesterogenic gene expression is reduced by OCA and is normalized by atorvastatin coadministration in humanized mice. A: qPCR analysis of mouse and human FXR-associated genes (CYP7A1, NR0B2 (SHP), SCARB1 (SR-B1), and SLC51B (OSTB)) in control (SCID) and PXB mouse livers, respectively. Significance was determined by Student’s t-test. Control: n = 5 mice per group; PXB: n = 8 or 9 mice per group. B: qPCR analysis of mouse cholesterol synthesis-associated genes in control (SCID) livers. Significance was determined by Student’s t-test. n = 5 mice per group. C: qPCR analysis of human cholesterol synthesis-associated genes in PXB livers. Significance was determined by Student’s t-test. n = 5 mice per group. D: qPCR analysis of human LDLR vs. CYP7A1 in PXB livers. E: Western blot analysis of LDLR, pSREBP2, and mSREBP2 in PXB livers treated with vehicle, OCA, ATV, or ATV + OCA. F: Western blot analysis of LDLR, pSREBP2, and mSREBP2 in PXB livers treated with vehicle, OCA, ATV, or ATV + OCA.
activation of SREBP-2. Consistent with this, Western blot analysis revealed significantly lower levels of mSREBP-2 in response to OCA treatment (Fig. 4E, F and supplemental Fig. 3B). Protein expression of the SREBP-2 target gene LDLR was also reduced. Similarly, serum levels of PCSK9, an inhibitor of LDLR and another target of SREBP-2, were slightly reduced (supplemental Fig. 3C), although this effect was not sufficient to rescue LDL-C. Thus, reduced hepatic LDLR protein expression likely contributed to OCA-induced increases in serum LDL-C. Coadministration of atorvastatin rescued SREBP-2 activation and LDLR protein expression (Fig. 4E, F and supplemental Fig. 3B). Together, these data suggest that OCA treatment leads to suppression of LDL-C uptake via reduced hepatic LDLR expression, which can be rescued with atorvastatin coadministration.

**FXR-mediated increases in LDL-C are also elicited by nonsteroidal FXR agonists in humanized PXB mice**

Steroidal and nonsteroidal FXR agonists differ in their pharmacokinetic properties and tissue distribution, wherein the action of nonsteroidal agonists is thought to be limited to the gut (39–41). This distinction has been hypothesized to avoid increases in serum LDL-C levels upon administration in humans. We compared the lipid effects of OCA with two nonsteroidal FXR agonists, INT-2228 and INT-2231, in PXB mice (Fig. 5A) (42–44). PXB mice treated with previously reported effective doses of INT-2228 (0.3 mg/kg/day) (41, 43, 44, 56) and INT-2231 (30 mg/kg BID) (40, 42) were compared with vehicle-treated and OCA-treated (10 mg/kg/day) mice after a 2 week dosing regimen. Body weight remained stable throughout the study in all groups (supplemental Fig. 4).

Expression of the FXR target genes *CYP7A1* and *SHP* in PXB livers showed similar target engagement for OCA and nonsteroidal agonists (Fig. 5B). Given that OCA-induced *SHP* expression is independent of gut FXR signaling (22, 57), these data reveal that nonsteroidal agonists directly act on liver FXR at the doses administered in this study.

As in the previous PXB cohort, OCA treatment led to significantly increased levels of serum LDL-C (Fig. 5C and supplemental Table 2). Consistent with the effects of OCA, both INT-2228 and INT-2231 increased levels of LDL-C (Fig. 5C). Additionally, hepatic LDLR mRNA as well as protein expression levels and SREBP-2 activation were downregulated with nonsteroidal agonists, as seen with OCA treatment (Fig. 5D–F). Thus, steroidal and nonsteroidal FXR agonists elicit similar hepatic changes and lead to increased levels of serum LDL-C in humanized PXB mice.

**DISCUSSION**

The results of the current investigation demonstrate the following: 1) the PXB mouse exhibits a human lipoprotein profile at baseline; 2) FXR activation with OCA induces changes in circulating lipoproteins in PXB mice similar to that observed in clinical trials; 3) the increase in LDL-C with OCA is most likely due to an indirect effect of FXR-mediated repression of bile acid synthesis, leading to elevated hepatic cholesterol; 4) atorvastatin coadministration reduces LDL-C in OCA-treated chimeric mice; and 5) nonsteroidal FXR agonists have direct hepatic effects and increase circulating LDL-C in chimeric mice.

Identification of the pharmacologic lipid response to FXR activation has been elusive, in part because studies in preclinical models are confounded by fundamental differences in cholesterol metabolism among animal models and humans. Opposite to the effects in humans, studies in animal models have generally shown reductions in serum cholesterol in response to FXR agonists. Here, we sought to identify a translational rodent model that more accurately reproduced lipoprotein changes observed in humans treated with FXR agonists. Similar to observations in FRG chimeric mice (36), PXB mice displayed an approximately 2:1 LDL-C/HDL-C ratio at baseline and an overall serum lipoprotein profile that is highly similar to that of healthy humans (23). Administration of OCA in PXB mice resulted in reduced serum HDL-C and increased LDL-C concentrations with a modest net increase in total serum cholesterol, which is reminiscent of the effects described in humans (17, 23). To our knowledge, this study is the first demonstration of FXR-mediated lipoprotein cholesterol alterations in a chimeric mouse model.

The translational value of PXB chimeric mice was also evident when coadministration of atorvastatin and OCA prevented the increase in circulating LDL-C levels. These results successfully predicted the effects described in humans (53, 54) and complemented those findings by providing a plausible mechanism associated with intracellular cholesterol, SREBP-2 activity, and LDLR protein expression. Statins not only inhibit de novo cholesterol synthesis, but also stimulate LDLR protein expression, thereby contributing to a significant drop in circulating LDL-C levels. Therefore, these data suggest that reduced LDLR protein levels in the liver may be a critical factor leading to elevated circulating LDL-C concentrations in humans treated with FXR agonists. This mechanism is consistent with the clinical observation that FXR agonists reduce hepatic LDLR expression and serum LDL-C clearance (26, 27). The results of these studies raise interesting questions for further investigation. Given the reported effects of statins on the bile

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one-way ANOVA with Dunnett’s correction (vs. vehicle-treated group). n = 8 or 9 mice per group. D: Correlation between human LDLR mRNA levels and human *CYP7A1* mRNA levels in individual PXB mice. Linear regression shown with goodness of fit (*R*²) value. P value indicates that the slope is significantly nonzero. E: Western blot analysis of liver samples from PXB mice treated with vehicle, OCA (10 mg/kg/day), and/or atorvastatin (ATV; 10 mg/kg/day). Full-length precursor SREBP2 is designated pSREBP2, and its active, mature form is designated mSREBP2. F: Protein quantification of all biological replicates from Western blots from E and supplemental Fig. 3B. Signal is normalized to βACTIN. Significance was determined by one-way ANOVA with Sidak’s correction (vs. OCA-treated group). * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001; n.s., not significant.
Fig. 5. FXR-mediated increases in LDL-C are also elicited by nonsteroidal FXR agonists in humanized PXB mice. A: Chemical structure of OCA and the two nonsteroidal compounds, INT-2228 and INT-2231, used in this study. B: qPCR analysis of human FXR-associated genes in PXB mouse livers. Significance was determined by one-way ANOVA with Dunnett’s correction (vs. vehicle). n = 5 mice per group. C: Serum cholesterol measurements from PXB mice were determined by gel permeation HPLC. Shown are the cholesterol levels that changed from baseline in response to vehicle, OCA (10 mg/kg/day), and/or atorvastatin (ATV; 10 mg/kg/day) treatment. Significance was determined by one-way ANOVA with Dunnett’s correction (vs. vehicle). n = 5 or 6 mice per group. D: Liver human LDLR gene expression levels of PXB mice measured by qPCR. Significance was determined by one-way ANOVA with Dunnett’s correction (vs. vehicle). n = 5 mice per group. E: Western blot analysis of liver samples from PXB mice treated with vehicle, OCA, INT-2228, and INT-2231. Five biological replicates are shown for each group. F: Protein quantification of Western blots from E. Levels of indicated proteins were normalized to levels of βACTIN. Significance was determined by Student’s t-test. n = 5 mice per group. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.
acid synthetic pathway (58, 59), and since the bile acid and cholesterol metabolic pathways intersect directly, further study of statin-FXR interactions in a translational model such as the PXB mouse is warranted.

Additional OCA-dependent pathways contributing to changes in HDL-C/LDL-C levels may also include repression of human APOA1 (60), upregulation of human cholesterol ester transfer protein (CETP) (61), or upregulation of mouse Insig2a (62). APOA1 mRNA was reduced by about 20% in PXB mice treated with OCA (supplemental Fig. 5A). This reduction may combine with the well-documented increase in SR-B1 expression to reduce levels of HDL-C in humans treated with OCA. CETP can also regulate the HDL-C/LDL-C ratio; however, human CETP mRNA levels were exceedingly low (average Ct value of 36.5) at baseline and, importantly, were not induced by OCA (supplemental Fig. 5B), indicating that this protein did not contribute to cholesterol changes in PXB mice. In line with this result, studies in hamsters, which express endogenous CETP, have produced mixed results, suggesting that additional OCA-dependent pathways exist (21, 29, 63, 64). Similar to the results for CETP, we found that INSIG2 is not induced with OCA in PXB mice (supplemental Fig. 5C). Thus, decreased levels of active SREBP2 in PXB livers are most likely a direct consequence of the negative feedback associated with increased intracellular cholesterol with FXR activation.

The expected consequence of reducing bile acid synthesis by FXR-mediated CYP7A1 repression is an increase in circulating total levels of cholesterol, as demonstrated in multiple clinical trials (1). However, empirical proof of this concept in the preclinical setting has been limited to a few studies in dog and hamster (64, 65), and, accordingly, the underlying mechanism is largely unclear. Within this context, PXB mice provide novel translational and mechanistic insights. Namely, the observation that intracellular levels of cholesterol increase in chimeric mice, but not in control mice, treated with FXR agonists is likely at the core of species-specific OCA responses.

The results of this study indicate that metabolic pathways initiated by FXR are different in humanized chimeric and control mice, with the notable distinction that hepatocellular cholesterol is increased in chimeric mice treated with OCA. An important feature of chimeric mice that may contribute to this observation is the impaired gut-liver signaling in the current model, i.e., human FGFR4/β-klotho is thought not to recognize mouse FGF19, which leads to increased hepatic CYP7A1 expression and bile acid synthesis in FRG chimeric mice (36, 50, 66). Upregulated bile acid synthesis in chimeric mice may therefore amplify the effects of OCA-induced repression of CYP7A1 and its downstream consequences on cholesterol metabolism. Notably, lack of gut-liver signaling in chimeric mice supports the viewpoint that the cholesterol effects of OCA are intrinsic to human hepatocytes, as repopulation in chimeric mice was sufficient to accurately recapitulate the human effects of OCA on circulating lipoproteins.

Regulation of intracellular hepatic cholesterol levels is complex, and differences among animal models are poorly understood. Apart from the classical pathway controlled by CYP7A1, alternative pathways of bile acid synthesis exist (e.g., CYP27A1) that are less dependent on FXR (1) and may play an important role in differentiating human and mouse responses. For example, alternative bile acid excretion pathways in mice appear to play a larger role in shaping the murine bile acid pool (67). A careful measurement of bile acid pool size, content, and fecal bile and cholesterol levels in chimeric mice is needed to elucidate the contribution of alternative excretion as a major factor in species-specific bile acid metabolism and cholesterol excretion in response to FXR agonists. Similar to excretion, intracellular levels of cholesterol alter efflux into the circulation through activation of liver X receptor and its targets, which include channels that load cholesterol in circulating HDL particles. However, this pathway is not likely to substantially contribute to changes in cholesterol levels in chimeric mice, as it would in principle trigger a futile cycle given that serum HDL-C is reduced in response to OCA.

The emergence of clinically relevant nonsteroidal FXR compounds has been fueled by a desire to develop agonists with improved chemical properties, pharmacokinetics, and target specificity. It is thought that the effects of OCA on circulating lipids are primarily caused by the cholesterol/bile acid scaffold of the compound, as well as its preferential accumulation in the liver. Additionally, it has been proposed that the distribution and actions of non-steroid-based FXR agonists are limited to the secretion of FGF19 from the intestine, i.e., “gut-restricted,” and thus are not associated with hepatic FXR activation that is responsible for the effects on cholesterol metabolism (40, 41). However, apart from a few published abstracts summarizing the effects of nonsteroidal FXR agonists in short-term (<2 weeks), small-scale clinical trials (40, 41), no comparative benchmarking clinical data are available that distinguish nonsteroidal agonists. Therefore, the assertion that nonsteroidal compounds have fewer effects on circulating lipids is for the moment anecdotal.

We compared the effects of OCA and two nonsteroidal FXR agonists in early development. The results show that both classes of compounds promote similar increases in circulating LDL-C. The similar intracellular responses also demonstrate that nonsteroidal FXR agonists directly act on hepatocytes in vivo (44). The doses of nonsteroid agonists used in the current experiments have been reported to be effective in rodent models of NASH (41, 68), while the effects in humans of the nonsteroid FXR agonists are reportedly devoid of effects on circulating lipids (40, 41). It is currently unclear whether nonsteroid FXR agonists are effective as therapeutic agents when administered at doses which localize in the intestine and act solely by release of FGF19. However, one would expect that FGF19-mediated suppression of CYP7A1 and bile acid synthesis would ultimately mirror the effects of OCA on circulating LDL-C. This view is supported by recent data in monkeys and humans, in which the FGF19 analog NGM282 increases circulating LDL-C (69), an effect that is reversible by a statin or inhibition of PCSK9 (69, 70). Therefore, the preclinical
evidence in this study combined with emerging clinical data on FGF19 signaling strongly support the notion that the increase in LDL-C is a class effect of FXR agonists, due to a direct effect on hepatic FXR and potentially an indirect intestine-mediated FGF19 effect.

In conclusion, our study highlights that the pattern of human cholesterol metabolism with FXR agonists is not adequately modeled in conventional preclinical rodent species. Given the pervasive body of literature demonstrating cholesterol-lowering effects of FXR activation in nonhuman species, our current data suggest that the metabolic changes due to FXR in vitro and in animal models should be interpreted cautiously in the context of their translational significance to human pathophysiology.

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