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CYP3A Activity and Expression in Nonalcoholic Fatty Liver Disease

Sarah J. Woolsey, Sara E. Mansell, Richard B. Kim, Rommel G. Tirona, and Melanie D. Beaton

Divisions of Clinical Pharmacology (S.J.W, S.E.M, R.B.K, R.G.T.) and Gastroenterology (M.D.B), Departments of Medicine (S.J.W, S.E.M, R.B.K, R.G.T., M.D.B.) and Physiology and Pharmacology (S.J.W, R.B.K, R.G.T.), Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is the leading cause of liver disease in the Western world, given its association with obesity, type 2 diabetes, and dyslipidemia. Medications are widely used in NAFLD to manage comorbid conditions, and there is significant interest in developing new drug therapies to treat the disease. Despite this, little is known about the effects of NAFLD on drug metabolism. We examined the activity and expression of the major drug-metabolizing enzyme subfamily, CYP3A, in subjects with NAFLD as well as in mouse and cellular models. CYP3A activity was determined in healthy volunteers and subjects with biopsy-proven NAFLD by oral midazolam phenotyping and measurement of plasma 4β-hydroxycholesterol, an endogenous metabolic biomarker. *CYP3A4* transcriptional activity, metabolic activity, and expression were also assessed in a mouse and cellular model of NAFLD. Subjects with nonalcoholic steatohepatitis (NASH) had 2.4-fold higher plasma midazolam levels compared with controls. Plasma 4β-hydroxycholesterol was 51% and 37% lower than controls in subjects with simple steatosis and NASH, respectively. Fibrosis was associated with 57% lower plasma 4β-hydroxycholesterol levels than controls. Furthermore, hepatic CYP3A4 mRNA expression in NASH was 69% lower than control livers. *CYP3A4* gene luciferase activity in the livers of NAFLD mice was 38% lower than that of controls. Lipid-loaded Huh7 human hepatoma cells had a 38% reduction in CYP3A4 activity and 80% lower CYP3A4 mRNA expression compared with the control. CYP3A activity is reduced in human NAFLD in addition to mouse and in vitro cell models of the disease.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in the Western world, affecting 20%–35% of the general adult population and 70%–90% of obese individuals (Browning et al., 2004; Bedogni et al., 2005). Given its close association with the metabolic syndrome and increased risk of cardiovascular disease, many NAFLD patients are prescribed a variety of medications to manage these associated conditions (Stepanova and Younossi, 2012). Although the liver is the primary site of drug metabolism, little is known about the effect of NAFLD on this process. With the current lack of approved pharmacologic treatments for NAFLD, much of the current focus of therapy for this condition has been in managing comorbid conditions. If significant differences in drug metabolism are present in NAFLD, this may have implications not only for dosing and administration of currently used medications but also for the development of new therapies targeting hepatic steatosis and fibrosis.

There is a paucity of information on the influence of NAFLD on the in vivo activity of major hepatic drug-metabolizing pathways. A key pathway involves CYP3A enzymes (CYP3A4 and CYP3A5), which act in the intestine and liver. CYP3A4 is responsible for the oxidative metabolism of more than 50% of all drugs, including those widely prescribed in NAFLD such as 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins), calcium channel blockers, thiazolidinediones, and sulfonylureas (Guengerich, 1999). Interindividual variability in hepatic CYP3A enzyme activity can reach 100-fold (Lin and Lu, 2001). This highly variable enzyme activity has been largely attributed to environmental factors (Burk and Wojnowski, 2004; Wilkinson, 2005) and genetic polymorphisms including reduced activity *CYP3A4*22* (Wang et al., 2011) and the inactivating allele *CYP3A5*3* (Kuehl et al., 2001).

In the setting of cirrhosis, there is clear in vivo evidence for reduced hepatic CYP3A activity, which contributes to decreased drug dose requirements (Verbeeck, 2008). However, in NAFLD with simple steatosis (SS) and nonalcoholic steatohepatitis (NASH), in vivo CYP3A activity has not been evaluated. A small number of ex vivo studies using archived livers have been published but findings are conflicting, reporting increased (Niemelä et al., 2000), decreased (Donato et al., 2006, 2007), or unchanged (Kolwankar et al., 2007; Fisher et al., 2009) hepatic CYP3A4 protein expression in NAFLD. Moreover, those studies that noted decreased CYP3A4 protein expression differed with respect to whether CYP3A4 mRNA was also reduced (Niemelä et al., 2000; Fisher et al., 2009). In a study of donated human type 2 diabetic liver, where NAFLD has a prevalence of 50%, hepatic CYP3A4 expression was reduced (Dostalek et al., 2011). Taken together, a majority of studies to date suggest that NAFLD is associated with reduced hepatic CYP3A activity; however, the data are heterogeneous and this finding has not yet been demonstrated in vivo.

In this study, we directly examined CYP3A drug metabolism activity in patients with biopsy-proven NAFLD as well as in both mouse and

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ABBREVIATIONS: 4β-OHC, 4β-hydroxycholesterol; HOMA IR, homeostatic model assessment of insulin resistance; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MDZ, midazolam; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAFLD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; SS, simple steatosis.

cell culture models of hepatic steatosis. We demonstrate, for the first time, that in vivo CYP3A activity is decreased in NAFLD.

Materials and Methods

In Vivo CYP3A Activity Phenotyping. The short-acting benzodiazepine, midazolam (MDZ), is oxidatively metabolized by CYP3A4 and CYP3A5 (Gorski et al., 1994). MDZ pharmacokinetic phenotyping is a widely used method to assess in vivo CYP3A activity (Lin et al., 2001). After an overnight fast, a group of 10 subjects with biopsy-proven NAFLD and a cohort of 20 healthy control subjects collected from previous studies reported by Woolsey et al. (submitted) and Gong et al. (2012) received an oral microdose (100 μg) of MDZ (1 mg/ml; Sandoz, Boucherville, Quebec, Canada) as an aqueous solution. Blood was collected 3 hours after drug administration for plasma MDZ concentration analysis. 4β-Hydroxycholesterol (4β-OHC) is a cholesterol metabolite formed by CYP3A4/CYP3A5 and is an endogenous biomarker for in vivo CYP3A activity (Diczfalusy et al., 2011). Fasting plasma was obtained from the healthy control subjects (n = 20) and subjects with biopsy-proven NAFLD (n = 30) for 4β-OHC level analysis. Histologic NAFLD stage was categorized as SS or NASH, according to the nonalcoholic fatty liver disease activity score (NAS), which includes steatosis (0–3), hepatic inflammation (0–3), and hepatocellular ballooning (0–2). Patients were categorized as having NASH if their NAS was $\geq$3 with a ballooning score of $\geq$1. SS was determined as total NAS of $<$3 or $\leq$3 with a ballooning score of 0. Hepatic fibrosis was scored separately (0–4) (no fibrosis = 0 and fibrosis = $\geq$1). Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA IR). These studies conformed to the ethical guidelines of the 1975 Declaration of Helsinki and were approved by the Human Subjects Research Ethics Board at the University of Western Ontario. All study participants provided informed written consent.

Genotyping. Single nucleotide polymorphisms associated with altered CYP3A activity were genotyped by TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA) for *CYP3A4*22* (rs35599367), *CYP3A5*3* (rs776746), peroxisome proliferator activating receptor α (*NR1C1,* rs4253728), and cytochrome P450 oxidoreductase *POR*28* (rs1057868). Patatin-like phospholipase domain-containing protein 3 (*PNPLA3,* rs738409) gene variation associated with hepatic steatosis was similarly determined.

Human Liver Tissues. Liver samples used for gene expression (mRNA) analyses were obtained by biopsy from subjects with NAFLD (n = 17; mean age 46 years; 10 men, 7 women; 3 SS, 14 NASH) as reported by Beaton et al. (2013), whereas normal human liver samples (n = 9; mean age 45 years; 3 men, 6 women) were obtained through the Liver Tissue Cell Distribution System (Minneapolis, MN; funded by National Institutes of Health Contract N01DK70004/HHSN267200700004C). Control livers were chosen as those without hepatic steatosis after Oil Red O histologic staining.

Drug, Metabolite, and Endogenous Biomarker Analysis. Plasma and samples from cell culture studies were analyzed for levels of MDZ and its CYP3A-catalyzed primary metabolite, 1-hydroxymidazolam, by liquid chromatography–tandem mass spectrometry (LC-MS/MS) according to our previous report (Woolsey et al., submitted). 4β-OHC levels in plasma were measured after picolinic acid derivatization and LC-MS/MS analysis according to the method of Honda et al. (2010) and detailed in our previous report (Woolsey et al., submitted).

Animal Studies. Female 5-week-old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, MA). Mice were fed a normal standard diet (2018 Teklad Global 18% protein rodent diet; Harlan Laboratories, Madison, WI) or a high-fat diet (TD.88137 Adjusted Calories Diet, 42% from fat; Harlan Laboratories) for 4 weeks. Human CYP3A4 reporter gene activity in the liver was determined in mice after hydrodynamic, tail-vein delivery (25 μg of DNA in 2 ml saline administered over 10 seconds) of a *CYP3A4* gene luciferase plasmid (CYP3A4-XREM-Luc) or a promoterless reporter (pGL3 Basic; Promega, Madison, WI) with correction for transfection efficiency with a Renilla luciferase vector (2 μg, pRL-CMV; Promega). The CYP3A4-XREM-Luc plasmid containing the proximal promoter (–362/+53) and distal xenobiotic response element (XREM; –7836/–7208) inserted in pGL3 Basic (Promega) was prepared previously (Tirona et al., 2003). Twenty-four hours after injection, livers were harvested and homogenized for analysis by the Dual-Luciferase assay (Promega). Liver segments were fixed and embedded in paraffin for staining with hematoxylin/eosin and trichrome or frozen in optimal cutting temperature for Oil Red O staining. This study protocol was approved by the University of Western Ontario Animal Use Subcommittee.

Cell Culture Studies. Huh7 human hepatoma cells (Japan Health Sciences Foundation, Tokyo, Japan) were cultured in high glucose Dulbecco's modified Eagle's medium (Lonza, Walkersville, MD) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 50 U/ml penicillin (Invitrogen), and 50 μg/ml streptomycin (Invitrogen) and were incubated at 37°C in 5% CO_2. Before the experiments, Huh7 cells were grown 3 weeks postconfluence with media changed routinely every 2 to 3 days. To induce steatosis, Huh7 cells were treated with 600 μM fatty acids (2:1 ratio of oleic and palmitic acids; Sigma-Aldrich, St. Louis, MO) in serum free media containing 1% fatty acid–free bovine serum albumin (Sigma-Aldrich) for 24 hours using a modified protocol (Sivertsson et al., 2010). Lipid accumulation was determined by Nile red staining and confocal fluorescence microscopy. Cell viability was assessed 24 hours after lipid loading using a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. To determine CYP3A4 metabolic activity, Huh7 cells were exposed to 1 μg/ml MDZ (ThermoFisher Diagnostix, Mississauga, Ontario, Canada) in Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with 12.5 mM HEPES and 5 mM glucose. After a 3-hour incubation, cell culture media were collected for analysis of 1-hydroxymidazolam concentration by LC-MS/MS as described above.

Gene Expression Analysis. RNA from liver samples and Huh7 cells was extracted using TRIzol (Invitrogen) and cDNA synthesized using MultiScribe reverse transcriptase (Applied Biosystems, Carlsbad, CA) with random hexamers. RNA quality and concentration was determined using an Agilent Bioanalyzer (RNA 600 Nano kit; Agilent, Santa Clara, CA) and a NanoVue Plus spectrophotometer (GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada). Relative mRNA expression of CYP3A4, CYP2E1, mCyp2e1, and mCyp3a11 were determined by SYBR Green–based quantitative polymerase chain reaction (ABI Prism 7700; Applied Biosystems). We used the following primer sequences: human CYP3A4, 5′-CAGGAGGAAATTGATGCAGTTTT-3′ (forward) and 5′-TCAAGATACTC-CATCTGTAGCACAGT-3′ (reverse); human CYP2E1, 5′- CCCAATCACCC-TGTCAATTT-3′ (forward) and 5′-GACCACCAGCACAACTCTGA-3′ (reverse); mouse Cyp2e1, 5′-CCTGGTGGAGGAGCTCAAAA-3′ (forward) and 5′-TGTTGAAGAGAATATCCGCAATGA-3′ (reverse); and mouse Cyp3a11, 5′-CTTTCCTTCACCCTGCATTCC-3′ (forward) and 5′-CTCATCCTGCAG-TTTTTTCTGGAT-3′ (reverse). Reactions were performed in triplicate for each sample and gene expression was normalized to 18S ribosomal RNA (TaqMan Gene Expression Assay; Applied Biosystems).

Statistical Analysis. Values are expressed as means ± S.E.M. or Tukey box plots. Differences between experimental groups were evaluated using an unpaired, two-tailed t test or a one-way analysis of variance with the Dunnett test. Differences were considered significant at the $P < 0.05$ level. All analysis was performed using GraphPad Prism software (version 5.0; GraphPad Software Inc., La Jolla, CA).

Results

CYP3A Activity and Expression Are Decreased in NAFLD. We examined in vivo CYP3A activity using oral MDZ phenotyping and plasma 4β-OHC biomarker level analysis. Control subjects (n = 20) were tested with both MDZ and 4β-OHC tests. MDZ phenotyping and 4β-OHC plasma level was determined in 10 and 30 subjects with biopsy-proven NAFLD, respectively. Subject demographics are summarized in Table 1. Neither healthy control nor NAFLD study subjects were taking CYP3A4-interacting medications at the time of study participation (Supplemental Tables 1 and 2). All NAFLD subjects and 17 of 20 control subjects consented to genetic analysis. There were no significant differences in the frequencies of allele carriers associated with CYP3A activity, MDZ pharmacokinetics, or plasma 4β-OHC levels among study groups (Table 1). We found that mean MDZ concentrations were 2.4-fold greater ($P < 0.0001$) in subjects with NASH (n = 9) compared with control subjects (Fig. 1A). The single subject with SS had 2.5-fold higher MDZ levels than controls (Fig. 1A). This result suggests that MDZ was not as readily metabolized in NASH

TABLE 1

Subjects phenotyped for CYP3A activity with MDZ and 4β-OHC tests

Characteristic	Control MDZ/4β-OHC (n = 20)	NAFLD MDZ (n = 10)	NAFLD 4β-OHC (n = 30)
Age, yr (range)	43 (28–58)	51 (27–63)	49 (27–69)
Sex			
Men	7	5	19
Women	13	5	11
Body mass index (range)	24 (19–30)	35 (28–45)	33 (23–45)
HOMA IR (range)	—	3.5 (1.7–6.5)	3.1 (1–9.6)
NAFLD stage[a]			
SS	—	1	7
NASH	—	9	23
Fibrosis[b]			
No fibrosis	—	1	6
Fibrosis	—	9	24
Allele carrier status[c]			
*CYP3A4*22*	17/0/0	9/1/0	24/6/0
*CYP3A5*3*	0/1/16	0/2/8	0/7/23
PPARα (rs4253728)	12/5/0	6/4/0	16/12/2
*POR*28*	10/6/1	5/5/0	13/14/3
PNPLA3 (rs738409)	—	2/7/1	9/14/7

[a]NASH was defined as an NAS [steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2)] ≥3 plus a hepatocellular ballooning score ≥1. SS was defined as an NAS <3 or an NAS ≤3 with a ballooning score of 0.
[b]Degree of fibrosis was categorized by histologic fibrosis score (0–4; no fibrosis = 0 and fibrosis = ≥1).
[c]Number of noncarriers/heterozygous carriers/homozygous carriers. For the control group, genotype was available for 17 of 20 subjects.

due to a decrease in CYP3A activity. NAFLD and healthy control subjects were also phenotyped for CYP3A activity using fasting plasma 4β-OHC level. NAFLD subjects had significantly lower mean 4β-OHC levels compared with control subjects (SS: 51% lower than control, $P < 0.001$; NASH: 37% lower than control, $P < 0.001$) (Fig. 1B), indicating decreased CYP3A activity. We separately examined the influence of hepatic fibrosis, *PNPLA3* genotype, and HOMA IR on plasma 4β-OHC levels. There were lower 4β-OHC levels in the presence of NAFLD fibrosis compared with control subjects (43% of control, $P < 0.0001$) (Fig. 1C). *PNPLA3* genotypes are associated with histologic severity of NAFLD (Sookoian and Pirola, 2011) and susceptibility to NASH (Zain et al., 2012). In the NAFLD cohort, carriers of the risk *PNPLA3* (rs738409) G allele tended to have lower 4β-OHC concentrations, although the association was not statistically significant (Supplemental Fig. 1A). Furthermore, insulin resistance, as assessed by HOMA IR, was not associated with plasma 4β-OHC levels among participants with NAFLD (Supplemental Fig. 1B). CYP3A4 mRNA expression level was determined in NAFLD biopsy samples and histologically normal, nonsteatotic archived livers. CYP3A4 mRNA levels were 69% lower in NASH biopsies (n = 14) than in control livers (n = 9; P = 0.059) (Fig. 1D). The amount of CYP3A4 mRNA was 60% lower in biopsies with SS (n = 3) than in control livers (n = 9); however, this difference was not statistically significant (P = 0.34) (Fig. 1D). In composite, results from both the MDZ and 4β-OHC phenotyping tests demonstrate that in vivo CYP3A activity is reduced in NAFLD. Fibrosis is associated with lower CYP3A enzyme function. Reduced in vivo CYP3A activity is associated with decreased hepatic CYP3A4 mRNA levels.

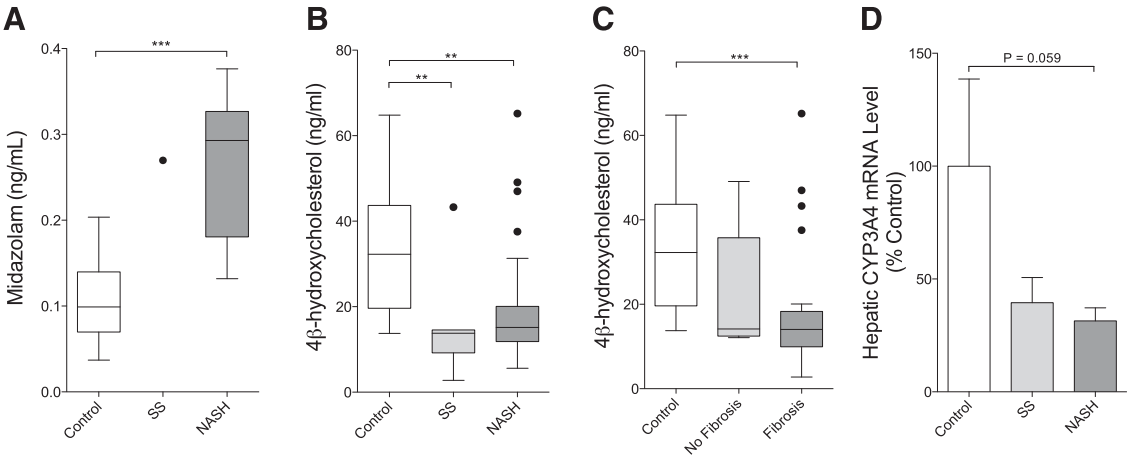

Fig. 1. CYP3A4 activity and expression in NAFLD. (A) Plasma MDZ concentrations 3 hours after oral MDZ microdose (100 μg) in healthy control (n = 20) and biopsy-proven NAFLD subjects (SS, n = 1; NASH, n = 9). Shown as Tukey box plots with median (line), 25 to 75 percentiles (box), and minimum/maximum values (whiskers). Statistical analysis by two-tailed t test (control versus NASH). (B) Fasting, plasma 4β-OHC concentrations in control (n = 20) and NAFLD subjects (SS, n = 7; NASH, n = 23). Statistical analysis by one-way ANOVA followed by the Dunnett test. (C) Plasma 4β-OHC concentrations in healthy controls (n = 20) and NAFLD subjects according to histologic assessment of fibrosis (no fibrosis, n = 6; fibrosis, n = 24). Statistical analysis by one-way ANOVA followed by the Dunnett test. (D) CYP3A4 mRNA expression in archived normal liver tissue (n = 9) and NAFLD liver biopsy samples (SS, n = 3; NASH, n = 14) compared using one-way ANOVA followed by the Dunnett test. Bars represent means with S.E.M. Gene expression was normalized to a commercial normal pooled human liver RNA sample. $^{**}P < 0.001$; $^{***}P < 0.0001$. ANOVA, analysis of variance.

Reduced CYP3A4 Transcriptional Activity in a Mouse Model of NAFLD. Female C57BL/6 mice were fed a high-fat diet for 4 weeks to induce NAFLD. SS was observed after hematoxylin and eosin, trichrome, and Oil Red O lipid staining of livers of mice fed a high-fat diet, whereas steatosis was absent in animals fed a normal diet (Fig. 2A). The livers of mice were in vivo transfected with a CYP3A4-XREM-Luc reporter plasmid or a pGL3 Basic control plasmid in conjunction with a normalizing Renilla luciferase vector, by the hydrodynamic tail-vein injection method. Hepatic CYP3A4 luciferase activity in the NAFLD mouse model was lower by 60% compared with mice on a normal diet (Fig. 2B). These results demonstrate that hepatic steatosis causes reduced liver *CYP3A4* transcriptional activity in an in vivo model of NAFLD.

CYP3A4 Activity and Expression Are Decreased in a NAFLD Cell Culture Model. Huh7 human hepatoma cells were incubated with and without fatty acids to induce steatosis. Lipid accumulation was confirmed using the neutral lipid stain, Nile red (Fig. 3, A and B). The fatty acid treatment did not to cause cytotoxicity up to concentrations of 600 μM, as determined by the MTT assay (Supplemental Fig. 2). Incubation of cells with MDZ (1 μg/ml) resulted in the appearance of the CYP3A metabolite, 1-hydroxymidazolam, in the culture media. The levels of 1-hydroxymidazolam in the fatty acid–treated Huh7 cells were lower by 38% compared with control cells (Fig. 3C), indicating reduced CYP3A enzyme activity in experimental steatosis. Furthermore, there was a significant decrease (reduction of 80%) in CYP3A4 mRNA expression in steatotic cells compared with control cells (Fig. 3D). These findings indicate that steatosis is associated with a reduction in CYP3A4 mRNA expression, leading to decreased enzyme activity in a cell culture model of NAFLD.

Discussion

With the global prevalence of NAFLD rising (Loomba and Sanyal, 2013), it is expected that this disease will become the number one indication for liver transplantation (Charlton et al., 2011). As such, the need for effective drug therapy to prevent disease progression is vital. Unfortunately, little is known about the effect of NAFLD on drug metabolism capacity, oral bioavailability, systemic exposure, and therapeutic response. The strongest evidence supporting altered drug metabolism relates to the well characterized induction of hepatic CYP2E1 expression and in vivo activity in NAFLD (Chalasani et al., 2003; Emery et al., 2003). CYP2E1 induction has been associated with enhanced susceptibility to acetaminophen bioactivation (to its reactive metabolite) and hepatotoxicity (Michaut et al., 2014). In this study, we also observed significantly increased CYP2E1 mRNA expression in both human NAFLD subjects and the cell culture model. In the mouse model of NAFLD, a trend toward an increased Cyp2e1 mRNA level was observed (Supplemental Fig. 3). Although there is evidence for CYP2E1 alterations in NAFLD, whether the expression and activity of the CYP3A subfamily is affected by NAFLD is not as clear. The in vivo activity of these primary drug-metabolizing enzymes in NAFLD has not been previously reported. In this study, we demonstrate that subjects with biopsy-proven NAFLD, phenotyped using an oral microdose of MDZ, have increased plasma MDZ concentrations compared with healthy control subjects (Fig. 1A). The validity of this simplified microdose and single-time-point sampling phenotyping strategy is supported by pharmacokinetic linearity of MDZ over a wide oral dose range (Halama et al., 2013) as well as a strong correlation between the 3-hour plasma concentration with area under the concentration-time curve (Lin et al., 2001; Woolsey et al., submitted). The observed 2.4-fold higher midazolam exposure in NASH compared with healthy subjects indicates moderately reduced CYP3A activity, given that the drug interaction with the potent CYP3A inhibitor ketoconazole results in a 16-fold increase in the oral midazolam area under the curve (Tsunoda et al., 1999).

We further assessed in vivo CYP3A activity by measuring plasma concentrations of 4β-OHC, a product of CYP3A-mediated metabolism of cholesterol (Diczfalusy et al., 2011). NAFLD patients had significantly lower 4β-OHC levels than controls, again indicating a decrease in CYP3A activity (Fig. 1B). Interestingly, CYP3A activity did not differ between NAFLD subjects with SS or NASH ($P = 0.4941$), despite studies demonstrating marked reduction in CYP3A4 expression and metabolic function in cultured human hepatocytes treated with inflammatory cytokines (Abdel-Razzak et al., 1993; Muntané-Relat et al., 1995). When examined independently from NAS, fibrosis, a marker of advanced NAFLD, was associated with significantly lower 4β-OHC levels compared with the control.

Plasma 4β-OHC levels are sensitive to the effects of CYP3A4 induction by drugs such as anticonvulsants (Bodin et al., 2001). However, the use of 4β-OHC as a biomarker for decreased CYP3A4 activity by enzyme inhibition with drugs may be limited due to the long half-life of this oxysterol, requiring weeks of inhibitor administration

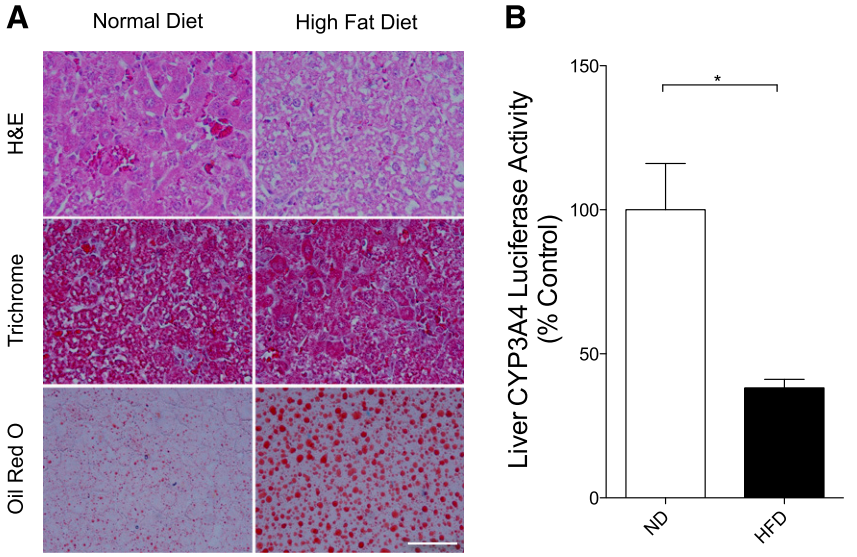

Fig. 2. CYP3A4 transcriptional activity in a NAFLD mouse model. (A) Representative H&E, trichrome, and Oil Red O staining of liver sections from adult mice fed a normal diet or a high-fat diet for 4 weeks. (B) Hepatic CYP3A4 luciferase reporter activity in mice after a normal diet ($n = 9$) or high-fat diet ($n = 5$). Values are presented as the mean and S.E.M. *$P <$ 0.05 (two-tailed t test). H&E, hematoxylin and eosin; HFD, high-fat diet; ND, normal diet. Bar, 20 μm.

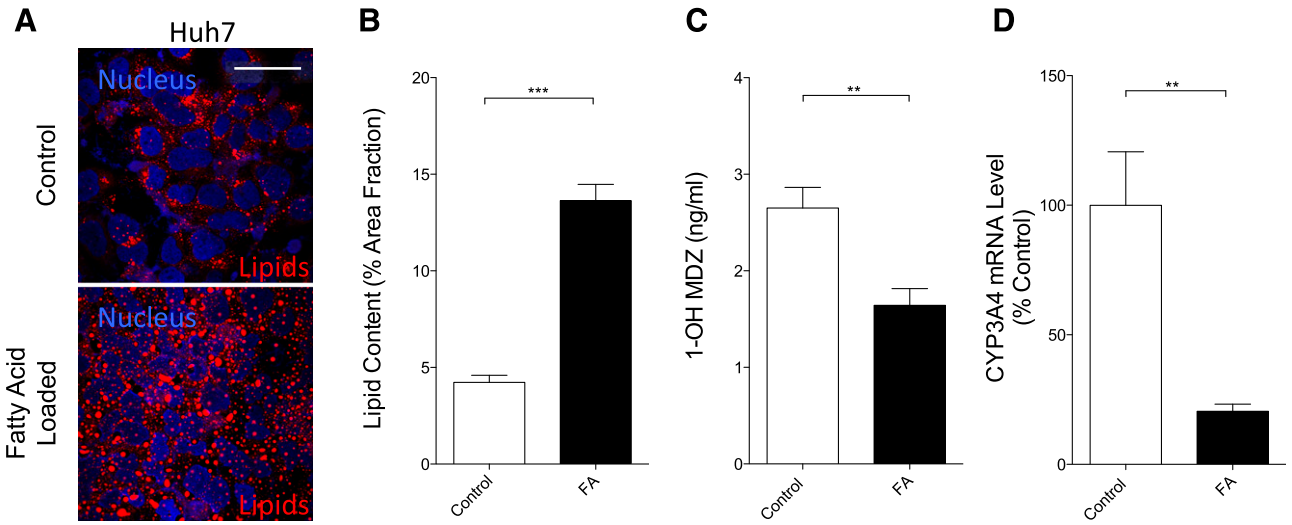

Fig. 3. CYP3A4 activity and expression in a cultured Huh7 human hepatoma cell NAFLD model. (A) Localization and accumulation of lipids in control and 24-hour, free fatty acid–treated (600 μM; oleate/palmitate, 2:1) Huh7 cells using Nile red lipid fluorescent stain. (B) Quantitative analysis of lipid accumulation within control and fatty acid–treated Huh7 cells by image analysis (ImageJ; National Institutes of Health, Bethesda, MD). (C) Accumulation of 1-hydroxymidazolam in the cell culture media after a 3-hour incubation with midazolam (1 μg/ml) in control (n = 6) and fatty acid–treated cells (n = 6). (D) Relative CYP3A4 mRNA expression in control (n = 9) and fatty acid–treated Huh7 cells (n = 9). Values are presented as means and S.E.M. $^{**}P < 0.001$; $^{***}P < 0.0001$ (two-tailed t test). FA, fatty acid. Bar, 50 μm.

for reductions in plasma levels to become apparent (Josephson et al., 2008). In the context of disease effects on CYP3A4 activity, our results in NAFLD, as well as those reported for Crohn's disease (Iwamoto et al., 2013), show that 4β-OHC may be a valid biomarker of reduced metabolic activity for chronic conditions. Plasma 4β-OHC levels are a reflection of CYP3A4 activity in the liver, as was demonstrated in a study of subjects treated with the enzyme inducer efavirenz (Meyer zu Schwabedissen et al., 2012). Systemic levels of this biomarker were increased, whereas no changes in intestinal CYP3A4 expression were observed. Our results implicate changes in liver CYP3A4 levels; however, the contribution of intestinal CYP3A4 activity to plasma 4β-OHC concentrations in NAFLD has not yet been formally evaluated.

There are some limitations to this study. Our findings of reduced CYP3A4 activity and expression in the mouse and cell culture models of NAFLD indicate that the observed increase in MDZ levels in NAFLD are at least partly a result of decreased hepatic activity. Larger pharmacokinetic studies using both oral and intravenous MDZ in NAFLD are required to define the metabolic changes that occur specifically in the liver and intestine.

For ethical reasons, liver biopsies could not be obtained from the control group to confirm absence of NAFLD. In this group, we considered anthropometric and serum biochemical indices for inclusion of healthy subjects into the control group. The average age of the control group was approximately 7 years younger than that of NAFLD subjects (Table 1). In our previous study of healthy subjects, we found that MDZ oral clearance was only reduced by 3% for every 10-year increase in age (Woolsey et al., submitted), whereas others have reported no effect of age on clearance (Gorski et al., 2003). We therefore do not consider the age difference between groups a significant contributor to the reduced CYP3A expression and activity.

To obtain further insight into the mechanisms of decreased in vivo CYP3A4 activity in NAFLD, additional experiments were performed in a diet-induced mouse NAFLD model. It is important to consider that CYP3A protein isoforms differ between rodents and humans. Specifically, mice express eight different active Cyp3a genes (Cyp3a11, Cyp3a13, Cyp3a16, Cyp3a25, Cyp3a41, Cyp3a44, Cyp3a57, and Cyp3a58), whereas adult humans express only two forms (CYP3A4 and genetically polymorphic CYP3A5) (Nelson et al., 2004). Furthermore, there are clear distinctions between mouse and human CYP3A gene regulation (Martignoni et al., 2006). Given the species difference in the expression and regulation of CYP3A genes, we delivered a *CYP3A4* gene promoter firefly luciferase reporter into the livers of mice with experimental hepatic steatosis. The advantages and effectiveness of this strategy are well documented in an in vivo experimental model with intact liver to study *CYP3A4* gene regulation (Schuetz et al., 2002; Tirona et al., 2003). Decreased liver CYP3A4 luciferase reporter activity in the mouse NAFLD model suggests that in the in vivo milieu of SS, there is reduced *CYP3A4* transcription (Fig. 2C). For comparison, we examined the expression of the predominant mouse hepatic Cyp3a11 enzyme in the SS model and found a trend toward lower (20% $\pm$ 6%, $P = 0.10$) mRNA expression levels in mice on a high-fat diet ($n = 6$) than in those on a normal diet ($n = 6$). In the context of previous reports, results in mouse models of NAFLD have been heterogeneous with some demonstrating decreased (Yoshinari et al., 2006; Ghose et al., 2011; Wahlang et al., 2014) or induced (Fisher et al., 2008; Spruiell et al., 2014) expression of Cyp3a11. Similarly, rat models of hepatic steatosis are conflicting, with some reporting decreased Cyp3a expression (Leclercq et al., 1998) and others showing higher levels (Ghoneim et al., 2015).

Finally, we examined CYP3A4 activity in a cultured human hepatoma cell model of steatosis. Huh7 cells were grown for weeks at confluence in these experiments because native expression and activity of CYP3A4 under these conditions is enhanced (Sivertsson et al., 2010). In fatty acid–induced steatotic Huh7 cells, we found a significant decrease in CYP3A4 activity similar to the results shown in NAFLD subjects in vivo (Fig. 3C). Reduced CYP3A4 activity was associated with decreased CYP3A4 mRNA levels (Fig. 3D), consistent with the findings of reduced CYP3A4 luciferase activity in the NAFLD mouse model.

The effects of inflammation and associated cytokines on hepatic drug metabolism gene expression are a probable mechanism for reduced CYP3A4 activity in NAFLD (Abdel-Razzak et al., 1993; Muntané-Relat et al., 1995; Pascussi et al., 2000; Jover et al., 2002). Indeed, inflammatory infiltration occurs in SS and NASH together with increased hepatic expression of inflammatory cytokines (Gadd et al., 2014). Inflammatory cytokines, acting through nuclear factor

κ-light-chain-enhancer of activated B cells, causes transrepression of the pregnane X receptor, a central transcription factor regulating CYP3A4 expression (Gu et al., 2006; Zhou et al., 2006). Moreover, the pregnane X receptor is downregulated by inflammatory cytokines (Pascussi et al., 2000) and its expression is reduced in human NASH (Bitter et al., 2014). Other mechanisms may be involved in the downregulation of CYP3A4 in NAFLD.

The clinical importance and drug development relevance of our findings of reduced CYP3A activity in NAFLD are potentially significant and remain to be further explored. Although CYP3A-metabolized medications such as some statins (which are commonly prescribed in patients with this condition) are safe, our finding that in vivo CYP3A metabolic activity is reduced in NAFLD leads one to ponder whether current drug dosing recommendations may need to be reevaluated in this population to ensure the best possible clinical outcomes for NAFLD patients with metabolic comorbidities. Indeed, we recently found that plasma 4β-OHC concentrations are associated with atorvastatin plasma levels during routine clinical care (DeGorter et al., 2013). Future investigations to determine the importance of altered drug metabolism in NAFLD, together with studies to elucidate the molecular mechanisms involved, will be required to provide additional insights into therapies and management of this important cause of liver disease.

Authorship Contributions

Participated in research design: Woolsey, Kim, Tirona, Beaton.

Conducted experiments: Woolsey, Mansell, Tirona, Beaton.

Performed data analysis: Woolsey, Tirona.

Wrote or contributed to the writing of the manuscript: Woolsey, Kim, Tirona, Beaton.

References

Abdel-Razzak Z, Loyer P, Fautrel A, Gautier JC, Corcos L, Turlin B, Beaune P, and Guillouzo A (1993) Cytokines down-regulate expression of major cytochrome P-450 enzymes in adult human hepatocytes in primary culture. *Mol Pharmacol* **44**:707–715.

Beaton MD, Chakrabarti S, Levstik M, Speechley M, Marotta P, and Adams P (2013) Phase II clinical trial of phlebotomy for non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* **37**: 720–729.

Bedogni G, Miglioli L, Masutti F, Tiribelli C, Marchesini G, and Bellentani S (2005) Prevalence of and risk factors for nonalcoholic fatty liver disease: the Dionysos nutrition and liver study. *Hepatology* **42**:44–52.

Bitter A, Rümmele P, Klein K, Kandel BA, Rieger JK, Nüssler AK, Zanger UM, Trauner M, Schwab M, and Burk O (2014) Pregnane X receptor activation and silencing promote steatosis of human hepatic cells by distinct lipogenic mechanisms. *Arch Toxicol* DOI: 10.1007/s00204-014-1348-x [published ahead of print].

Bodin K, Bretillon L, Aden Y, Bertilsson L, Broomé U, Einarsson C, and Diczfalusy U (2001) Antiepileptic drugs increase plasma levels of 4beta-hydroxycholesterol in humans: evidence for involvement of cytochrome p450 3A4. *J Biol Chem* **276**:38685–38689.

Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, and Hobbs HH (2004) Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* **40**:1387–1395.

Burk O and Wojnowski L (2004) Cytochrome P450 3A and their regulation. *Naunyn Schmiedebergs Arch Pharmacol* **369**:105–124.

Chalasani N, Gorski JC, Asghar MS, Asghar A, Foresman B, Hall SD, and Crabb DW (2003) Hepatic cytochrome P450 2E1 activity in nondiabetic patients with nonalcoholic steatohepatitis. *Hepatology* **37**:544–550.

Charlton MR, Burns JM, Pedersen RA, Watt KD, Heimbach JK, and Dierkhising RA (2011) Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. *Gastroenterology* **141**:1249–1253.

DeGorter MK, Tirona RG, Schwarz UI, Choi YH, Dresser GK, Suskin N, Myers K, Zou G, Iwuchukwu O, and Wei WQ, et al. (2013) Clinical and pharmacogenetic predictors of circulating atorvastatin and rosuvastatin concentrations in routine clinical care. *Circ Cardiovasc Genet* **6**:400–408.

Diczfalusy U, Nylén H, Elander P, and Bertilsson L (2011) 4β-Hydroxycholesterol, an endogenous marker of CYP3A4/5 activity in humans. *Br J Clin Pharmacol* **71**:183–189.

Donato MT, Jiménez N, Serralta A, Mir J, Castell JV, and Gómez-Lechón MJ (2007) Effects of steatosis on drug-metabolizing capability of primary human hepatocytes. *Toxicol In Vitro* **21**: 271–276.

Donato MT, Lahoz A, Jiménez N, Pérez G, Serralta A, Mir J, Castell JV, and Gómez-Lechón MJ (2006) Potential impact of steatosis on cytochrome P450 enzymes of human hepatocytes isolated from fatty liver grafts. *Drug Metab Dispos* **34**:1556–1562.

Dostalek M, Court MH, Yan B, and Akhlaghi F (2011) Significantly reduced cytochrome P450 3A4 expression and activity in liver from humans with diabetes mellitus. *Br J Pharmacol* **163**:937–947.

Emery MG, Fisher JM, Chien JY, Kharasch ED, Dellinger EP, Kowdley KV, and Thummel KE (2003) CYP2E1 activity before and after weight loss in morbidly obese subjects with nonalcoholic fatty liver disease. *Hepatology* **38**:428–435.

Fisher CD, Jackson JP, Lickteig AJ, Augustine LM, and Cherrington NJ (2008) Drug metabolizing enzyme induction pathways in experimental non-alcoholic steatohepatitis. *Arch Toxicol* **82**:959–964.

Fisher CD, Lickteig AJ, Augustine LM, Ranger-Moore J, Jackson JP, Ferguson SS, and Cherrington NJ (2009) Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of nonalcoholic fatty liver disease. *Drug Metab Dispos* **37**:2087–2094.

Gadd VL, Skoien R, Powell EE, Fagan KJ, Winterford C, Horsfall L, Irvine K, and Clouston AD (2014) The portal inflammatory infiltrate and ductular reaction in human nonalcoholic fatty liver disease. *Hepatology* **59**:1393–1405.

Ghoneim RH, Ngo Sock ET, Lavoie JM, and Piquette-Miller M (2015) Effect of a high-fat diet on the hepatic expression of nuclear receptors and their target genes: relevance to drug disposition. *Br J Nutr* **113**:507–516.

Ghose R, Omoluabi O, Gandhi A, Shah P, Strohacker K, Carpenter KC, McFarlin B, and Guo T (2011) Role of high-fat diet in regulation of gene expression of drug metabolizing enzymes and transporters. *Life Sci* **89**:57–64.

Gong IY, Crown N, Suen CM, Schwarz UI, Dresser GK, Knauer MJ, Sugiyama D, DeGorter MK, Woolsey S, and Tirona RG, et al. (2012) Clarifying the importance of CYP2C19 and PON1 in the mechanism of clopidogrel bioactivation and in vivo antiplatelet response. *Eur Heart J* **33**: 2856–2864.

Gorski JC, Hall SD, Jones DR, VandenBranden M, and Wrighton SA (1994) Regioselective biotransformation of midazolam by members of the human cytochrome P450 3A (CYP3A) subfamily. *Biochem Pharmacol* **47**:1643–1653.

Gorski JC, Vannaprasaht S, Hamman MA, Ambrosius WT, Bruce MA, Haehner-Daniels B, and Hall SD (2003) The effect of age, sex, and rifampin administration on intestinal and hepatic cytochrome P450 3A activity. *Clin Pharmacol Ther* **74**:275–287.

Gu X, Ke S, Liu D, Sheng T, Thomas PE, Rabson AB, Gallo MA, Xie W, and Tian Y (2006) Role of NF-kappaB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. *J Biol Chem* **281**: 17882–17889.

Guengerich FP (1999) Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol* **39**:1–17.

Halama B, Hohmann N, Burhenne J, Weiss J, Mikus G, and Haefeli WE (2013) A nanogram dose of the CYP3A probe substrate midazolam to evaluate drug interactions. *Clin Pharmacol Ther* **93**:564–571.

Honda A, Miyazaki T, Ikegami T, Iwamoto J, Yamashita K, Numazawa M, and Matsuzaki Y (2010) Highly sensitive and specific analysis of sterol profiles in biological samples by HPLC-ESI-MS/MS. *J Steroid Biochem Mol Biol* **121**:556–564.

Iwamoto J, Saito Y, Honda A, Miyazaki T, Ikegami T, and Matsuzaki Y (2013) Bile acid malabsorption deactivates pregnane X receptor in patients with Crohn's disease. *Inflamm Bowel Dis* **19**:1278–1284.

Josephson F, Bertilsson L, Böttiger Y, Flamholc L, Gisslén M, Ormaasen V, Sönnerborg A, and Diczfalusy U (2008) CYP3A induction and inhibition by different antiretroviral regimens reflected by changes in plasma 4beta-hydroxycholesterol levels. *Eur J Clin Pharmacol* **64**: 775–781.

Jover R, Bort R, Gómez-Lechón MJ, and Castell JV (2002) Down-regulation of human CYP3A4 by the inflammatory signal interleukin-6: molecular mechanism and transcription factors involved. *FASEB J* **16**:1799–1801.

Kolwankar D, Vuppalanchi R, Ethell B, Jones DR, Wrighton SA, Hall SD, and Chalasani N (2007) Association between nonalcoholic hepatic steatosis and hepatic cytochrome P-450 3A activity. *Clin Gastroenterol Hepatol* **5**:388–393.

Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, and Hall SD, et al. (2001) Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat Genet* **27**:383–391.

Leclercq I, Horsmans Y, Desager JP, Delzenne N, and Geubel AP (1998) Reduction in hepatic cytochrome P-450 is correlated to the degree of liver fat content in animal models of steatosis in the absence of inflammation. *J Hepatol* **28**:410–416.

Lin JH and Lu AY (2001) Interindividual variability in inhibition and induction of cytochrome P450 enzymes. *Annu Rev Pharmacol Toxicol* **41**:535–567.

Lin YS, Lockwood GF, Graham MA, Brian WR, Loi CM, Dobrinska MR, Shen DD, Watkins PB, Wilkinson GR, and Kharasch ED, et al. (2001) In-vivo phenotyping for CYP3A by a single-point determination of midazolam plasma concentration. *Pharmacogenetics* **11**:781–791.

Loomba R and Sanyal AJ (2013) The global NAFLD epidemic. *Nat Rev Gastroenterol Hepatol* **10**:686–690.

Martignoni M, Groothuis GM, and de Kanter R (2006) Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin Drug Metab Toxicol* **2**:875–894.

Meyer zu Schwabedissen HE, Oswald S, Bresser C, Nassif A, Modess C, Desta Z, Ogburn ET, Marinova M, Lütjohann D, and Spielhagen C, et al. (2012) Compartment-specific gene regulation of the CAR inducer efavirenz in vivo. *Clin Pharmacol Ther* **92**:103–111.

Michaut A, Moreau C, Robin MA, and Fromenty B (2014) Acetaminophen-induced liver injury in obesity and nonalcoholic fatty liver disease. *Liver Int* **34**:e171–e179.

Muntané-Relat J, Ourlin JC, Domergue J, and Maurel P (1995) Differential effects of cytokines on the inducible expression of CYP1A1, CYP1A2, and CYP3A4 in human hepatocytes in primary culture. *Hepatology* **22**:1143–1153.

Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, and Nebert DW (2004) Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics* **14**:1–18.

Niemelä O, Parkkila S, Juvonen RO, Viitala K, Gelboin HV, and Pasanen M (2000) Cytochromes P450 2A6, 2E1, and 3A and production of protein-aldehyde adducts in the liver of patients with alcoholic and non-alcoholic liver diseases. *J Hepatol* **33**:893–901.

Pascussi JM, Gerbal-Chaloin S, Pichard-Garcia L, Daujat M, Fabre JM, Maurel P, and Vilarem MJ (2000) Interleukin-6 negatively regulates the expression of pregnane X receptor and constitutively activated receptor in primary human hepatocytes. *Biochem Biophys Res Commun* **274**:707–713.

Schuetz E, Lan L, Yasuda K, Kim R, Kocarek TA, Schuetz J, and Strom S (2002) Development of a real-time in vivo transcription assay: application reveals pregnane X receptor-mediated induction of CYP3A4 by cancer chemotherapeutic agents. *Mol Pharmacol* **62**:439–445.

Sivertsson L, Ek M, Darnell M, Edebert I, Ingelman-Sundberg M, and Neve EP (2010) CYP3A4 catalytic activity is induced in confluent Huh7 hepatoma cells. *Drug Metab Dispos* **38**:995–1002.

Sookoian S and Pirola CJ (2011) Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease. *Hepatology* **53**:1883–1894.

Spruiell K, Jones DZ, Cullen JM, Awumey EM, Gonzalez FJ, and Gyamfi MA (2014) Role of human pregnane X receptor in high fat diet-induced obesity in pre-menopausal female mice. *Biochem Pharmacol* **89**:399–412.

Stepanova M and Younossi ZM (2012) Independent association between nonalcoholic fatty liver disease and cardiovascular disease in the US population. *Clin Gastroenterol Hepatol* **10**:646–650.

Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, and Gonzalez FJ, et al. (2003) The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat Med* **9**:220–224.

Tsunoda SM, Velez RL, von Moltke LL, and Greenblatt DJ (1999) Differentiation of intestinal and hepatic cytochrome P450 3A activity with use of midazolam as an in vivo probe: effect of ketoconazole. *Clin Pharmacol Ther* **66**:461–471.

Verbeeck RK (2008) Pharmacokinetics and dosage adjustment in patients with hepatic dysfunction. *Eur J Clin Pharmacol* **64**:1147–1161.

Wahlang B, Song M, Beier JI, Cameron Falkner K, Al-Eryani L, Clair HB, Prough RA, Osborne TS, Malarkey DE, and Christopher States J, et al. (2014) Evaluation of Aroclor 1260 exposure in a mouse model of diet-induced obesity and non-alcoholic fatty liver disease. *Toxicol Appl Pharmacol* **279**:380–390.

Wang D, Guo Y, Wrighton SA, Cooke GE, and Sadee W (2011) Intronic polymorphism in CYP3A4 affects hepatic expression and response to statin drugs. *Pharmacogenomics J* **11**:274–286.

Wilkinson GR (2005) Drug metabolism and variability among patients in drug response. *N Engl J Med* **352**:2211–2221.

Yoshinari K, Takagi S, Yoshimasa T, Sugatani J, and Miwa M (2006) Hepatic CYP3A expression is attenuated in obese mice fed a high-fat diet. *Pharm Res* **23**:1188–1200.

Zain SM, Mohamed R, Mahadeva S, Cheah PL, Rampal S, Basu RC, and Mohamed Z (2012) A multi-ethnic study of a PNPLA3 gene variant and its association with disease severity in non-alcoholic fatty liver disease. *Hum Genet* **131**:1145–1152.

Zhou C, Tabb MM, Nelson EL, Grün F, Verma S, Sadatrafiei A, Lin M, Mallick S, Forman BM, and Thummel KE, et al. (2006) Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest* **116**:2280–2289.

Address correspondence to: Dr. Melanie D. Beaton, Division of Gastroenterology, Department of Medicine, London Health Sciences Centre, University Hospital, 339 Windermere Rd., London, ON N6A 5A5, Canada. E-mail: melanie.beaton@lhsc.on.ca