Association Between Nonalcoholic Hepatic Steatosis and Hepatic Cytochrome P-450 3A Activity

DHANASHRI KOLWANKAR,* RAJ VUPPALANCHI,*,‡ BRIAN ETHELL,§ DAVID R. JONES,* STEVEN A. WRIGHTON,§ STEPHEN D. HALL,* and NAGA CHALASANI*,‡

*Division of Clinical Pharmacology and ‡Division of Gastroenterology/Hepatology, Department of Medicine, Indiana University School of Medicine; and §Department of Drug Disposition, Lilly Research Laboratories, Indianapolis, Indiana

Background & Aims: **Comorbidities associated with nonalcoholic fatty liver often require therapy with medications (eg, statins) metabolized by cytochrome P-450 3A (CYP3A). There is significant interindividual variability in CYP3A expression. However, human studies that systematically examined the relationship between hepatic steatosis and hepatic CYP3A activity are lacking.** ***Methods:*** **The relationship of hepatic CYP3A activity with several variables including hepatic steatosis, CYP3A4 protein content, CYP3A4 mRNA expression, CYP3A5 genotype, and its mRNA expression was investigated in human liver samples (n = 49). CYP3A activity was quantified from liver microsomes by using testosterone as a probe, and hepatic steatosis was defined to be present if >5% of hepatocytes had large globules of intracellular fat displacing the nucleus.** ***Results:*** **The mean ± standard error hepatic CYP3A activity of the study group was 3156 ± 2794 pmol · min^{-1} · mg^{-1} of protein, and it was not associated with age, gender, medicinal use, CYP3A5 or pregnane xenobiotic receptor mRNA expression, or CYP3A5 genotype. Twenty-four liver samples with steatosis had significantly lower hepatic CYP3A activity than 25 liver samples without steatosis (1978 ± 299 vs 4287 ± 659 pmol · min^{-1} · mg^{-1} of protein; *P* = .003). This difference persisted even after controlling for relevant covariates in the multivariate analysis (*P* = .04). However, CYP3A4 protein content was not different between the 2 groups (6 ± 1.3 vs 8.5 ± 2.2 pmol/mg protein; *P* = .3). There was a significant negative relationship between severity of steatosis and hepatic CYP3A activity (*P* = .01).** ***Conclusions:*** **Hepatic steatosis is associated with decreased hepatic CYP3A activity in humans via post-translational mechanism. Further studies are needed to confirm our findings.**

Nonalcoholic fatty liver disease (NAFLD) is a metabolic disorder characterized by hepatic accumulation of free fatty acids and triglycerides.[1] Its spectrum ranges from benign hepatic steatosis to nonalcoholic steatohepatitis (NASH) associated with lobular inflammation, necrosis, progressive fibrosis, and cirrhosis.[2,3] The prevalence of NAFLD appears to be increasing with the prevalence of obesity. An estimated one third of the subjects from a multiethnic, population-based sample in Dallas County, Texas had hepatic steatosis when hepatic triglyceride content was measured by using proton magnetic resonance spectroscopy.[4] Obesity, dyslipidemia, insulin resistance, diabetes, and hypertension are commonly associated with NAFLD.[5–7] These comorbidities frequently necessitate therapy with drugs that are metabolized by the enzyme cytochrome P-450 (CYP3A).[8] Several studies suggest that patients with NAFLD have altered hepatic cytochrome P-450 2E1 activity.[9–11] Recent data from cultured human hepatocytes incubated with long chain free fatty acid showed a general down-regulation of P450s involved in drug metabolism in fat-overloaded hepatocytes.[12] In some animal models of steatosis induced by nutritional manipulation, hepatic fat accumulation was associated with a significant decrease in CYP3A protein and activity.[13] However, human studies that systematically examined the relationship between hepatic steatosis and hepatic CYP3A activity are lacking.

The CYP3A subfamily of enzymes present in the intestinal epithelium and liver mediate the first pass and systemic metabolism of more than 50% of the drugs that undergo oxidative biotransformation.[14] The CYP3A subfamily comprises *CYP3A4, CYP3A5, CYP3A7,* and more recently discovered *CYP3A43*. CYP3A7 is primarily a fetal enzyme, and CYP3A43's contribution to CYP3A activity is thought to be negligible.[15] CYP3A4 is the predominant form contributing to most of the hepatic CYP3A activity in humans. However, the wide pharmacokinetic variability of drugs that are metabolized by CYP3A is thought to be due to its variable expression. Prior studies have shown that xenobiotic-induced regulation of CYP3A4 enzyme is mediated through pregnane xenobiotic receptor (PXR).[14–17] In a collection of human liver biopsy samples, Wolbold et al[18] have shown a good correlation between PXR, CYP3A4 mRNA, and protein expression, suggesting that PXR regulates the expression of CYP3A4, or that expression of these genes is co-regulated. Further variability in CYP3A activity could be due to polymorphically expressed CYP3A5 enzyme.[19] The presence of *CYP3A5* wild-type (wt) allele (*CYP3A5*1*) is associated with significantly higher expression of CYP3A5 protein and likely contributes substantially to the total CYP3A activity.[20] However, the studies that evaluated the contribution of CYP3A5 to total CYP3A activity are limited and have yielded mixed results.[21–27] *CYP3A5*3* is the primary mutant allele of *CYP3A5*, and the single nucleotide polymorphism in intron 3 results in the early termination of the mRNA (CYP3A5 SV1) along with some amount of correctly spliced mRNA. CYP3A5*6 and *7 are

Abbreviations used in this paper: CYP3A, cytochrome P-450 3A; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PCR, polymerase chain reaction; PXR, pregnane xenobiotic receptor; SV, splice variant; wt, wild-type.

1542-3565/07/$32.00
doi:10.1016/j.cgh.2006.12.021

other mutant alleles known not to express functional CYP3A5 enzyme.[28]

In the current study, we examined the relationship of hepatic CYP3A activity with hepatic steatosis, CYP3A5 genotype, and PXR expression in a large group of human liver samples.

Methods

Samples of human liver tissue were obtained from individuals with no known liver disease. These samples were obtained from Indiana University or University of Pittsburgh and were acquired with institutional approval during the cadaveric solid organ donation process from individuals who had negative serology for human immunodeficiency virus, HBV, HCV, and syphilis and lacked significant alcohol consumption. From each consented donor, more than 50 g of wedged liver tissue was collected. Although demographic, clinical, and medicinal data were available on all subjects, details of smoking and diet were lacking. This study was approved by our Institutional Review Board.

Tissue Preparation

Histologic assessment under light microscopy was performed by an experienced single hepatopathologist on the liver tissue slides stained with hematoxylin-eosin. Macrovesicular steatosis was assessed, and fatty liver was defined to be present if more than 5% of the hepatocytes had large globules of intracellular fat displacing the nucleus.[29,30] RNA was isolated by using RNeasy Mini Kit (Qiagen, Valencia, CA). The quality and quantity of RNA were measured on Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA) along with RNA 600 Nano LabChip Kit (Caliper Technologies, Hopkinton, MA). RNA samples with good 28S to 18S ratio (2:1) were used for quantitation of mRNA. Hepatic microsomes were prepared and stored at −80°C until used.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction

Total RNA was reverse transcribed to cDNA by using the Reverse Transcription system (Promega Corp, Madison, WI). Real-time reverse transcriptase polymerase chain reaction (PCR) methods from prior published literature were used to measure the amount of CYP3A5 wt and CYP3A5 splice variant (SV1; predominant splice variant associated with CYP3A5*3 genotype),[16] CYP3A4, and hPXR mRNA.[18] For CYP3A5*1 and *3, the PCR mixture contained Taqman Universal Master Mix reagent (Applied Biosystems, Foster City, CA), forward and reverse primer (400 nmol/L each), and 100 nmol/L of fluorescent probe. Amplification and detection were performed by using Icycler (Biorad Laboratories, Hercules, CA), and the PCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds and 60°C for CYP3A5 wt and 62°C for CYP3A5 SV1 for 1 minute. The PCR mixture for CYP3A4 and hPXR contained Platinum Quantitative PCR supermix UDG (Invitrogen, Carlsbad, CA), forward and reverse primer (200 nmol/L each for CYP3A4 and 300 nmol/L for hPXR), and the PCR conditions were 50°C for 2 minutes and then 42 cycles of 95°C for 15 seconds and 60°C for 1 minute. Detection of CYP3A4 and hPXR was done with SYBR green Core Reagents (Molecular Probes, Inc, Eugene, OR). The calibration curves were generated by serial dilutions of CYP3A5 wt and SV1, CYP3A4, and hPXR. Data were normalized to 18S mRNA expression.[31]

Genotyping

CYP3A5*3 and *6 genotyping was performed by allele-specific real-time PCR by using the method of Hiratsuka et al[32] with minor modifications. The assay was adapted to a real-time SYBR green assay (Bio-Rad iCycler; Biorad Laboratories) by eliminating the fluorescent labeled Taqman probe and modifying the PCR mix as follows. The reaction mixture consisted of Platinum Quantitative PCR Supermix-UDG (Invitrogen Corporation), the common and allele-specific primers described by Hiratsuka et al (final concentration, 400 nmol/L), SYBR Green I (1:100,000 final dilution; Molecular Probes, Inc), fluorescein (1 nmol/L final concentration), and 10–30 ng of DNA template.[32] Control DNA samples of known genotypes were included in each run as quality controls. The amplification detection protocol was 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 5 minutes, 45 cycles of 95°C for 10 seconds and 60°C for 35 seconds, and a melt curve at the end of 80 cycles starting at 60°C and increasing by 0.5°C per cycle. The CYP3A5*7 allele was detected on the basis of a method previously published by Eap et al.[33]

CYP3A Activity

CYP3A activity was measured by using testosterone as a probe, and formation of 6β-hydroxytestosterone was quantified from liver microsomal samples.[34,35] In brief, 200 μmol/L testosterone was incubated with human liver microsomes (0.2 mg total protein/mL), 100 mmol/L phosphate buffer pH = 7.4, and 2 mmol/L reduced nicotinamide adenine dinucleotide phosphate for 5 minutes. The reaction was stopped with 2 mmol/L ascorbic acid in methanol. 6β-hydroxytestosterone was quantified from the supernatant with liquid chromatography/mass spectrometry by using corticosterone as the internal standard.

Western Blot Analysis

Proteins in human liver microsomes were resolved on a 10% Tris-HCl polyacrylamide gel, transferred to a nitrocellulose membrane (Bio-Rad), and blotted with a CYP3A4/CYP3A7 antibody, which does not detect CYP3A5 (catalog # 458234; BD Biosciences, Woburn, MA). cDNA-expressed CYP3A4 (catalog # 456207; BD Biosciences) was used as a standard. The immunoreactive bands were detected with an alkaline phosphatase–conjugated rabbit α-mouse IgG (BD Biosciences) by treating the membrane with nitroblue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt developing solution. Optical densities of the bands on the membrane were converted to quantitative numbers by using the Kodak electrophoresis documentation and analysis system 290 (EDAS 290) and Kodak 1D image analysis software (Eastman Kodak, Rochester, NY). A standard curve (0.13–2.00 μmoles) was run on the same gel as the liver samples. The amount of CYP3A4 per milligram of microsomal protein was estimated by comparing the band densities of each liver sample with the standard curve of the cDNA-expressed CYP3A4.

Statistical Analysis

Descriptive statistics such as mean, standard error, and percentages were used to characterize the cohort. Comparisons