Experimental Nonalcoholic Steatohepatitis Increases Exposure to Simvastatin Hydroxy Acid by Decreasing Hepatic Organic Anion Transporting Polypeptide Expression

John D. Clarke, Rhiannon N. Hardwick, April D. Lake, Mark J. Canet, and Nathan J. Cherrington

Department of Pharmacology and Toxicology, University of Arizona, Tucson, Arizona

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ABSTRACT

Simvastatin (SIM)-induced myopathy is a dose-dependent adverse drug reaction (ADR) that has been reported to occur in 18.2% of patients receiving a 40- to 80-mg dose. The pharmacokinetics of SIM hydroxy acid (SIMA), the bioactive form of SIM, and the occurrence of SIM-induced myopathy are linked to the function of the organic anion transporting polypeptide (Oatp) hepatic uptake transporters. Genetic polymorphisms in *SLCO1B1*, the gene for human hepatic OATP1B1, cause decreased elimination of SIMA and increased risk of developing myopathy. Nonalcoholic steatohepatitis (NASH) is the most severe form of nonalcoholic fatty liver disease, and is known to alter drug transporter expression and drug disposition. The purpose of this study was to assess the metabolism and disposition of SIM in a diet-induced rodent model of NASH.

Rats were fed a methionine- and choline-deficient diet for 8 weeks to induce NASH and SIM was administered intravenously. Diet-induced NASH caused increased plasma retention and decreased biliary excretion of SIMA due to decreased protein expression of multiple hepatic Oatps. SIM exhibited increased volume of distribution in NASH as evidenced by increased muscle, decreased plasma, and no change in biliary concentrations. Although Cyp3a and Cyp2c11 proteins were decreased in NASH, no alterations in SIM metabolism were observed. These data, in conjunction with our previous data showing that human NASH causes a coordinated downregulation of hepatic uptake transporters, suggest that NASH-mediated transporter regulation may play a role in altered SIMA disposition and the occurrence of myopathy.

Introduction

Statins are a widely prescribed and relatively safe class of drugs used to treat hyperlipidemia and reduce the risk of heart disease and stroke (Bruckert et al., 2005; Fernandez et al., 2011). The most common adverse drug reactions (ADRs) related to statin therapy are the musculoskeletal toxicities, broadly termed myopathy, which range from myalgia to potentially fatal rhabdomyolysis (Thompson et al., 2006). Although myopathy is usually not life-threatening, the cost to the healthcare system is concerning because of the resources required for management of this adverse event (Fernandez et al., 2011). In addition, the cost to a patient's health is potentially great because it has been estimated that up to 60% of elderly patients prescribed a statin will discontinue therapy within 2 years, at least partially due to myopathy, thus placing them at increased risk of heart disease and stroke (Fernandez et al., 2011). Simvastatin (SIM) is one statin for which multiple risk factors

for myopathy have been identified, including dose, genetic variation in hepatic uptake transporter organic anion transporting polypeptide-1B1 (OATP1B1), and hepatic impairment (Thompson et al., 2006; Link et al., 2008; Fernandez et al., 2011; Watson and McLeod, 2011).

Pharmacogenetic analyses have identified a single nucleotide polymorphism (SNP) (rs4149056) in *SLCO1B1*, the gene for OATP1B1, that increases systemic exposure of simvastatin hydroxy acid (SIMA), the bioactive metabolite of SIM, and increases the risk of SIM-induced myopathy (Wilke et al., 2012). It was shown that the presence of two rs4149056 alleles caused a 221% increase in SIMA area under the curve (AUC) and a 200% increase in SIMA maximum plasma concentration (C_{max}) (Pasanen et al., 2006). In patients taking 80 mg of simvastatin daily, the odds ratio for myopathy per copy of rs4149056 was 4.5, with greater than 60% of the myopathy cases attributable to the SNP (Link et al., 2008). These rs4149056-associated changes in SIMA pharmacokinetics and increased risk of myopathy led to publication of dose adjustment guidelines in the context of this SNP by the Clinical Pharmacogenomics Implementation Consortium (Wilke et al., 2012).

Although genetic variation clearly plays a role in the occurrence of ADRs, other factors such as liver diseases are

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ABBREVIATIONS: ADR, adverse drug reaction; AUC, area under the curve; Cl, clearance; ERK1, extracellular signal-regulated kinase 1; MCD, methionine and choline deficient; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OATP, organic anion transporting polypeptide; SIM, simvastatin; SIM-d6, simvastatin-d6; SIMA, simvastatin hydroxy acid; SIMA-d6, simvastatin-d6 hydroxy acid; SNP, single nucleotide polymorphism; V_d , volume of distribution.

also known to alter drug pharmacokinetics and contribute to the occurrence of ADRs (Verbeeck, 2008; Merrell and Cherrington, 2011; Gandhi et al., 2012; Naik et al., 2013). Liver diseases that impair hepatic function may require dose adjustments to maintain drug concentrations within the therapeutic window and avoid ADRs (Delcò et al., 2005; Verbeeck, 2008). Nonalcoholic steatohepatitis (NASH) is one such liver disease that is becoming increasingly prevalent (up to 17% of US adults) and has been shown to cause alterations in drug transporters and drug disposition (McCullough, 2006; Ali and Cusi, 2009; Hardwick et al., 2011, 2012; Lake et al., 2011; Merrell and Cherrington, 2011). We previously showed that altered expression of multiple hepatic Oatp transporters in rats caused increased plasma concentrations and decreased biliary excretion of sulfobromophthalein, a dye used in liver function tests (Fisher et al., 2009). We have also shown that NASH in human liver samples caused a coordinated downregulation of hepatic uptake transporters (Lake et al., 2011). These findings in NASH and the results presented here suggest that other Oatp substrates, such as the statins, may also exhibit altered disposition in NASH patients and increase the risk of statin-induced myopathy.

Materials and Methods

SIM and SIMA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Simvastatin-d6 (SIM-d6) and simvastatin-d6 hydroxy acid (SIMA-d6) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Ammonium acetate and urethane were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile, methanol, and high-performance liquid chromatography-grade water were purchased from Thermo Fisher Scientific (Pittsburg, PA). The Oasis HLB μ Elution 96-well plates were purchased from Waters Corporation (Milford, MA).

Animal Treatment. Eight-week-old male Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, Indiana). Rats were maintained in 12-hour light and 12-hour dark cycles in a University of Arizona animal facility and were allowed water and standard laboratory diet ad libitum during the 1-week acclimation period. All animal treatments were performed in accordance with protocols approved by the University of Arizona's Institutional Animal Care and Use Committee. After acclimation, rats were divided into two groups and fed either a methionine- and choline-deficient (MCD) diet or methionine- and choline-replete (control) diet from Dyets Inc. (Bethlehem, PA). After 8 weeks of feeding, animals were anesthetized with a 1.2 g/kg intraperitoneal dose of urethane (5 ml/kg). During anesthesia, animal body temperature was monitored and maintained at 37°C with a TCAT-2V heat pad from Physitemp Instruments Inc (Clifton, NJ). The carotid artery and jugular vein were cannulated with PE-50 polyethylene tubing and the common bile duct was cannulated with PE-10 polyethylene tubing from Braintree Scientific (Braintree, MA). SIM lactone was administered into the carotid artery at a dose of 1 mg/kg (5 ml/kg) in 10% ethanol, 45% propylene glycol, 15% glycerine, and 30% water. Blood samples were collected from the jugular vein into heparinized tubes 0, 2, 10, 20, 40, 60, 90, and 120 minutes after SIM administration, and bile was collected at 15-minute intervals over the course of the 120-minute experiment. Whole blood was centrifuged at 10,000 rpm for 1 minute to collect plasma. After the final blood and bile collections, the rats were euthanized, and liver, kidney, and soleus tissues were collected. A slice of liver was taken and fixed in formalin at 4°C overnight followed by 70% ethanol and embedded in paraffin for hematoxylin and eosin staining. Kidney, soleus, and the remaining liver tissue were flash-frozen in liquid nitrogen and stored at -80°C.

SIM Quantification. SIM and SIMA quantification was performed according to a previously published protocol with slight modifications

for extraction from other biologic matrices (Yang et al., 2005). For plasma and bile samples, 25 μ l of each sample was mixed with 10 μ l of 100 mM ammonium acetate (pH 4.5) and 2 μ l of internal standards simvastatin-d6 and simvastatin-d6 hydroxy acid (6.2 μ g/ml). Samples were centrifuged at 10,000 rpm for 5 minutes at 4°C. Ninety-six-well Waters Oasis HLB μ Elution plates were conditioned sequentially with 200 μ l of acetonitrile and 200 μ l of methanol followed by equilibration with 200 μ l of water. Thirty microliters of the supernatant was applied to a preconditioned and equilibrated Oasis HLB μ Elution plate (30 μ m). For tissues, an entire soleus or approximately 250 mg of liver or kidney was weighed and cut into small pieces with scissors. Tissues were homogenized in 600 μ l of 100 mM ammonium acetate (pH 4.5) with a Dounce homogenizer. The homogenate was transferred to a micro-centrifuge tube, vortexed, and frozen at -80°C to ensure complete lysis of the cells. Samples were then thawed and centrifuged at 10,000 rpm for 5 minutes at 4°C. Three-hundred microliters of the homogenate were removed, mixed with 2 μ l of internal standards simvastatin-d6 and simvastatin-d6 hydroxy acid (6.2 μ g/ml), and 250 μ l was applied to a preconditioned and equilibrated HLB plate. The above-mentioned volumes of prepared biologic matrices were applied to the plate followed by two sequential washes with 400 μ l of 5% methanol. Samples were eluted with 50 μ l of 95/5 (v/v) acetonitrile and water followed by dilution with 50 μ l of 1 mM methyl ammonium acetate (pH 4.5). Samples in the autosampler were maintained at 10°C, and 10 μ l was injected onto the column. Ultra-performance liquid chromatography was performed with a Waters Acquity system connected to a Waters Acquity BEH C18 1.7 μ m 2.1 $\times$ 50 mm column maintained at 40°C. An isocratic mobile phase consisting of 80% acetonitrile and 20% 1 mM methyl ammonium acetate (pH 4.5) was used to separate the compounds at a flow rate of 0.3 ml/min. The eluent from the column was delivered to a Waters Micromass Quattro Premier XE tandem mass spectrometer that operated in polarity switching mode to detect ions in both positive and negative mode. Compounds were quantified by multiple reaction monitoring of precursor-product ion transitions in positive mode at m/z 450 to 303 (SIM) and 456 to 285 (SIM-d6), and negative mode at 435 to 319 (SIMA) and 441 to 319 (SIMA-d6).

mRNA Analysis. RNA isolation and mRNA quantification were performed as previously described with specific probes for Cyp2c11, Cyp3a1/23, Oatp1b2, Oatp1a1, and Oatp1a4 (Hardwick et al., 2012).

Protein Analysis. Liver whole-cell lysates were collected as previously described (Hardwick et al., 2012). Membrane proteins were isolated from a portion of the whole-cell lysate by ultracentrifugation at 100,000g for 60 minutes. The supernatant was discarded, and the pellet was resuspended in 0.1 M Tris buffer with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN) added per the manufacturer's instruction. Sixty micrograms of whole-cell lysate or membrane preparation was reduced and denatured in Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) and 5% 2-mercaptoethanol at 37°C for 30 minutes. Samples were separated on 7.5% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The following antibodies were used to detect each protein: Cyp2c11 (ab3571; Abcam, Cambridge, MA), Cyp3a1 (sc-53246; Santa Cruz Biotechnology), Oatp1b2 (sc-376904; Santa Cruz Biotechnology), Oatp1a1 (OATP11-A; Alpha Diagnostics International, San Antonio, TX), Oatp1a4 (OATP21-A; Alpha Diagnosis International), extracellular signal-regulated kinase 1 (ERK1) (sc-93; Santa Cruz Biotechnology), and pan-cadherin (ab16505; Abcam). Pan-cadherin and ERK1 were used as loading control for blots from membrane and whole preparations, respectively. Images were captured using the Image Laboratory Imaging System (Bio-Rad Laboratories) and relative protein quantities were determined using ImageJ software (National Institutes of Health, Bethesda, MD).

Pharmacokinetics Analysis. The AUC was calculated using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). SIM clearance (Cl) was calculated using the dose, AUC, and body weight for each animal. SIM volume of distribution (V_d) was calculated by dividing the Cl value by the slope of the linear regression of the logarithm plasma concentration by time plot for each animal.

Statistical Analysis. A *t* test within GraphPad Prism software was used to determine differences between control and MCD groups. All data represent the mean $\pm$ S.E.M.

Results

Altered Disposition of SIM and SIMA in Diet-Induced NASH. After 8 weeks, animals on the MCD diet had characteristic pathologic features of NASH, including macrovesicular steatosis, fibrosis, and inflammation (Fig. 1), similar to previous reports (Lickteig et al., 2007; Hardwick et al., 2012). To determine how NASH affects hepatobiliary disposition of SIM and SIMA, concentrations of each compound were measured in plasma and bile over the course of the study. The plasma concentrations of SIM were reduced by more than 50% in animals with NASH (Fig. 2A), whereas the biliary excretion was not different between the two groups (Fig. 2B). For SIMA, NASH caused a 160% increase in plasma retention (Fig. 2C) with a concomitant 28% decrease in biliary excretion (Fig. 2D). Further investigation into the pharmacokinetic parameters of SIM indicated that the volume of distribution was increased 360% in NASH animals (Table 1), potentially due to increased muscle concentrations of SIM (Fig. 3). Within the kidney, SIMA concentrations were approximately 3-fold higher in animals who received the MCD diet (Fig. 3). The higher SIMA plasma concentrations (Fig. 2C) and lower liver concentrations (Fig. 3)

Fig. 1. MCD diet-induced NASH. Hematoxylin and eosin-stained formalin-fixed paraffin-embedded liver sections from rats fed an MCD diet for 8 weeks had characteristic features of NASH. Rats fed the control diet for 8 weeks had healthy livers with no evidence of NAFLD. Original magnification, $\times 20$.

Fig. 2. The effect of diet-induced NASH on SIM and SIMA disposition. Plasma samples were collected from the carotid artery at 2, 10, 20, 40, 60, 90, and 120 minutes, and bile samples were collected from the common bile duct every 15 minutes after intravenous administration of SIM in control (open circles, solid line) and MCD (open squares, dashed line) rats. The plasma (A) and bile (B) concentrations and AUCs of SIM were measured. The plasma (C) and bile (D) concentrations and AUCs of SIMA were measured. Bars represent the mean $\pm$ S.E.M. ($n = 5$ for each group). * $P < 0.05$; ** $P < 0.01$.

caused a 91% lower SIMA liver to plasma ratio in NASH animals, whereas this ratio for SIM did not reach statistical significance (Fig. 4). Together, these data indicate that NASH impaired the uptake of SIMA into the liver.

OATP Expression in Diet-Induced NASH. Hepatic Oatp uptake transporter mRNA and protein expression was measured by branched DNA and Western blot, respectively. Oatp1b2 and Oatp1a1 mRNA expression was significantly reduced by 52% and 46%, respectively, in NASH livers, whereas the reduction in Oatp1a4 mRNA did not reach statistical significance (Fig. 5A). At the protein level, expression of Oatp1b2, Oatp1a1, and Oatp1a4 was significantly reduced in NASH livers by 30%, 39%, and 83%, respectively (Fig. 5B). These data suggest that the decrease in hepatic uptake transporters is responsible for the NASH-induced increase in plasma concentrations, decreased biliary elimination, and decreased liver concentrations of SIMA.

SIM Metabolism Is Not Altered in NASH. To determine whether NASH altered the metabolism of SIM, we measured the mRNA and the protein expression of Cyp2c11 and Cyp3a1/23 and used the AUC values for SIM and SIMA in bile and plasma. Diet-induced NASH caused a dramatic 92% and 87% decrease in Cyp2c11 and Cyp3a1/23 mRNA, respectively (Fig. 6A). At the protein level, diet-induced NASH caused an 87% and 84% decrease in Cyp2c11 and Cyp3a1/23 protein, respectively (Fig. 6A). In spite of these changes in metabolism enzymes, our data do not indicate that SIM metabolism was altered in NASH because the decrease in P450 enzymes in NASH would lead to higher SIM concentrations in these animals, which is opposite to what was observed in the plasma (Fig. 2A). Metabolites other than SIM and SIMA have been reported in rodents (Ishigami et al., 2001) and although we did not directly measure any other metabolites, our data indicate that NASH did not induce differential metabolism because the sums of SIM and SIMA AUCs were not different between the treatment groups in plasma or bile (Fig. 6B).

Discussion

The incidence of NAFLD and NASH in the United States has increased in recent years and, as our understanding of the disease has grown, the clinical challenges facing this patient population have increased as well. For example, an increasing number of publications show that NASH alters the expression of hepatic transporters and the disposition of multiple xenobiotic substrates, potentially placing NASH into the category of liver diseases that impair hepatic function and require drug dose adjustments (Lickteig et al., 2007; Fisher et al., 2009; Lake et al., 2011; Merrell and Cherrington, 2011; Canet et al., 2012; Clarke and Cherrington, 2012; Hardwick et al., 2012). NAFLD patients are also at increased risk of developing and dying from cardiovascular disease and are thus frequently prescribed lipid-lowering drugs such as statins

Fig. 3. The effect of diet-induced NASH on SIM and SIMA tissue concentrations. At the 120-minute time point, liver, kidney, and soleus muscles were collected and homogenized and the concentrations of SIM (A) and SIMA (B) were measured. Data are shown as the amount of drug per gram of tissue. Data represent the mean $\pm$ S.E.M. ($n = 5$). * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

(Targher et al., 2005, 2006, 2007; Ekstedt et al., 2006; Fan, 2008; Ali and Cusi, 2009; Nseir et al., 2012). In light of our recent data showing decreased expression of hepatic uptake transporters in NASH (Fisher et al., 2009; Lake et al., 2011) and the fact that many of the statins rely heavily on these uptake transporters (Elsby et al., 2012), we undertook the present investigation to determine how an experimental model of NASH affects the metabolism and disposition of SIM and how this may influence the occurrence of statin-induced myopathy.

Active hepatic uptake of SIMA has been estimated to contribute at least 75% of the hepatic extraction ratio (Elsby et al., 2012). There is a high degree of overlap in substrate specificity among the OATP transporters; within the human liver, OATP1B1 and OATP1B3 are the main OATP isoforms responsible for drug uptake (Kalliokoski and Niemi, 2009). Many of the statins are

Fig. 4. Liver to plasma ratio of SIM and SIMA in control and diet-induced NASH rats. Liver to plasma ratios ($K_{p \text{ liver}}$) were calculated by dividing the plasma concentration at 120 minutes by the liver concentrations at 120 minutes. Data represent the mean $\pm$ S.E.M. ($n = 5$). ** $P < 0.01$.

TABLE 1

SIM plasma Cl and V_d

P values were calculated with the t test.

Pharmacokinetic Parameter	Control	MCD	P Value
<i>Mean $\pm$ S.E.M.</i>			
Cl (ml/min)	92 $\pm$ 8	132 $\pm$ 25	0.1702
V_d (ml)	3199 $\pm$ 445	11,570 $\pm$ 2760	0.0171

Fig. 5. mRNA and protein expression changes of rat hepatic Oatp uptake transporters in diet-induced NASH. (A) Oatp1b2, Oatp1a1, and Oatp1a4 mRNA expression is shown as relative light units (RLUs) per 10 µg of total RNA. (B) Oatp1b2 protein expression was normalized to pan-cadherin levels, whereas Oatp1a1 and Oatp1a4 protein expression was normalized to ERK expression. Data represent the mean $\pm$ S.E.M. ($n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

substrates for the different OATP isoforms (Kallikokoski and Niemi, 2009) but data showing that SIMA is a substrate for the OATPs have been challenging, potentially because of its physical properties and methodological difficulties (Elsby et al., 2012). Despite this, there is clear evidence that the *SLCO1B1* SNP rs4149056 increases SIMA exposure and the risk of SIM-induced myopathy (Voora et al., 2009; Niemi, 2010; Wilke et al., 2012). The overlap in substrate specificity creates a complex situation in which other OATPs that are fully functional can compensate for the genetic polymorphisms that affect the function of only one transporter. We have shown that human NASH potentially reduces this compensatory transport mechanism through a co-ordinated decrease in hepatic uptake transporter expression (Lake et al., 2011). The results from the current study show that

Fig. 6. NASH-associated changes in SIM metabolism enzymes and their impact on SIM metabolism. (A) Cyp2c11 and Cyp3a1/23 mRNA (RLU per 10 µg of total RNA) and protein levels (normalized to ERK1) were measured in rat livers ($n = 6$ per group). (B) The sum of SIM and SIMA AUCs in plasma and the sum of SIM and SIMA AUCs in bile ($n = 5$ per group). Data represent the mean $\pm$ S.E.M. ** $P < 0.01$; *** $P < 0.001$. RLU, relative light unit.

in the context of diet-induced NASH, there is reduced expression of multiple rodent hepatic Oatp isoforms leading to an increase in SIMA plasma retention, as indicated by increased plasma concentrations, decreased liver concentrations, and decreased biliary excretion. Other data from the literature support this mechanism of compensatory transport of statins by other uptake transporters. In Oatp1b2 knockout mice, the function of Oatp1a transporters in rodents has been suspected to compensate for the loss of Oatp1b2 (Degorté et al., 2012), which may explain why a previous report showed that SIMA did not have an altered liver to plasma ratio in Oatp1b2 knockout mice (Chen et al., 2008). As further evidence for this mechanism, it has been shown that knockout of the entire Oatp1a/1b cluster potentiated the increase in pravastatin plasma exposure compared with Oatp1b2 knockout alone (Zaher et al., 2008; Iusuf et al., 2012). These data indicate that NASH-specific alterations in uptake transporters may have a compounding effect on drug disposition and the occurrence of ADRs because of changes in multiple compensatory transporters.

Our current data also show that SIMA concentrations were higher in the kidneys of rats fed an MCD diet. The mechanism behind this change in disposition is currently unclear but will be the focus of future investigations for drugs that are highly dependent on renal elimination. For the current study, because statins are primarily dependent on hepatobiliary elimination (Shitara and Sugiyama, 2006), it is not anticipated that this change in kidney statin concentrations will influence the metabolism and disposition of SIM.

SIM is administered as the prodrug lactone that must be metabolized into SIMA to elicit its cholesterol-lowering properties. SIM can be metabolized into SIMA and other metabolites through both enzymatic and nonenzymatic pathways (Prueksaritanont et al., 1997). The enzymatic metabolism of SIM into SIMA and several other metabolites is reported to be carried out by the Cyp2c11 isoform in male rats and the Cyp3a isoform in female rats (Ishigami et al., 2001). Nonenzymatic hydrolysis from SIM lactone into SIMA has also been reported to be carried out by esterases and paraoxonases (Prueksaritanont et al., 1997; Neuvonen et al., 2006). In this study, we administered the lactone form of SIM and observed conversion into SIMA in both control and MCD diet-induced NASH animals, in which we observed a sharp decrease in Cyp2c11 and Cyp3a1/23 expression. If these changes in P450 enzyme levels affected the metabolism of SIM, then it would be expected that NASH animals would have much higher SIM levels and lower SIMA levels in plasma. Our data show exactly the opposite result, suggesting that P450-mediated metabolism does not account for the differences in SIM and SIMA concentrations observed in our study. In addition, our data indicate that SIM was not metabolized differentially in control and MCD animals into alternate metabolites since the sums of the AUCs for SIM and SIMA in plasma or bile were not different between the treatment groups. Changes in P450 expression and activity are common in liver diseases, and we have previously shown that the expression and activity of multiple human P450 isoforms are altered in human NASH. The mechanism behind these changes in P450s and impact of these changes in human populations requires further investigation. These data indicate that MCD diet-induced NASH did not alter the metabolism of SIM and, therefore, the differences observed in SIMA concentrations are a result of transporter-mediated processes.

SIM is a lipophilic compound that is not dependent on active transport to cross membrane barriers (Pasanen et al., 2006). The lipophilic nature of SIM has also been reported to contribute to its larger volume of distribution compared with SIMA (Prueksaritanont et al., 2005). In this study, we observed that MCD diet-induced NASH caused an increase in the volume of distribution for SIM. This change in volume of distribution was caused by SIM more readily partitioning into the muscle of NASH animals, thus decreasing the plasma concentrations without affecting the amount of SIM excreted into the bile. These results are particularly striking since SIM-induced myopathy is a dose-dependent ADR that occurs in the muscle, suggesting that the altered disposition of SIMA in NASH may increase the risk of SIM-induced myopathy. Although further investigation into the cause of this increased volume of distribution is needed, these data clearly indicate that NASH alters the disposition of SIM and may contribute to the occurrence of SIM-induced myopathy.

The work presented here further highlights the need for more directed investigation into how liver disease affects SIMA disposition and the occurrence of SIM-induced myopathy, especially since SIM and other statins have been proposed as treatment options for NAFLD and NASH (Nseir et al., 2012). To date, the small preliminary investigations for SIM have concluded that it is safe in patients with NAFLD or NASH. Unfortunately, these studies only considered SIM-induced hepatotoxicity but did not evaluate, nor did they have the statistical power to detect, an increased risk of myopathy in NASH (Abel et al., 2009; Nelson et al., 2009). Furthermore, one of these studies looked at NAFLD broadly defined as elevated liver enzyme levels in the absence of other kinds of liver diseases rather than NASH specifically. Multiple previous reports suggest altered drug disposition and dose-dependent ADRs may occur only following the development of NASH, highlighting the need for well designed studies that will examine the safety of SIM specifically in patients diagnosed with NASH (Fisher et al., 2009; Lake et al., 2011). Conducting these studies will be challenging due to the difficulty in obtaining a liver biopsy, which continues to be the only effective method of NASH diagnosis (Mofrad et al., 2003; Verbeeck, 2008). Collectively, the data shown here provide further impetus for the design of specific clinical trials that will evaluate the disposition of SIMA and the occurrence of SIM-induced myopathy in NASH patients.

Authorship Contributions

Participated in research design: Clarke, Hardwick, Cherrington.

Conducted experiments: Clarke, Hardwick, Lake, Canet.

Contributed new reagents or analytic tools: Hardwick.

Performed data analysis: Clarke.

Wrote or contributed to the writing of the manuscript: Clarke, Hardwick, Lake, Canet, Cherrington.

References

- Abel T, Fehér J, Dinya E, Eldin MG, and Kovács A (2009) Safety and efficacy of combined ezetimibe/simvastatin treatment and simvastatin monotherapy in patients with non-alcoholic fatty liver disease. *Med Sci Monit* 15:MS6–MS11.
- Ali R and Cusi K (2009) New diagnostic and treatment approaches in non-alcoholic fatty liver disease (NAFLD). *Ann Med* 41:265–278.
- Bruckert E, Hayem G, Dejager S, Yau C, and Bégaud B (2005) Mild to moderate muscular symptoms with high-dosage statin therapy in hyperlipidemic patients—the PRIMO study. *Cardiovasc Drugs Ther* 19:403–414.
- Canet MJ, Hardwick RN, Lake AD, Kopplin MJ, Scheffer GL, Klimecki WT, Gandolfi AJ, and Cherrington NJ (2012) Altered arsenic disposition in experimental non-alcoholic fatty liver disease. *Drug Metab Dispos* 40:1817–1824.
- Chen C, Stock JL, Liu X, Shi J, Van Deussen JW, DiMattia DA, Dullea RG, and de Morais SM (2008) Utility of a novel Oatp1b2 knockout mouse model for evaluating

- the role of Oatp1b2 in the hepatic uptake of model compounds. *Drug Metab Dispos* **36**:1840–1845.
- Clarke JD and Cherrington NJ (2012) Genetics or environment in drug transport: the case of organic anion transporting polypeptides and adverse drug reactions. *Expert Opin Drug Metab Toxicol* **8**:349–360.
- DeGorter MK, Urquhart BL, Gradhand U, Tirona RG, and Kim RB (2012) Disposition of atorvastatin, rosuvastatin, and simvastatin in oatp1b2^{-/-} mice and intra-individual variability in human subjects. *J Clin Pharmacol* **52**:1689–1697.
- Delcò F, Tchambaz L, Schlienger R, Drewe J, and Krähenbühl S (2005) Dose adjustment in patients with liver disease. *Drug Saf* **28**:529–545.
- Ekstedt M, Franzén LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, and Kechagias S (2006) Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* **44**:865–873.
- Elsby R, Hilgendorf C, and Fenner K (2012) Understanding the critical disposition pathways of statins to assess drug-drug interaction risk during drug development: it's not just about OATP1B1. *Clin Pharmacol Ther* **92**:584–598.
- Fan JG (2008) Impact of non-alcoholic fatty liver disease on accelerated metabolic complications. *J Dig Dis* **9**:63–67.
- Fernandez G, Spatz ES, Jablecki C, and Phillips PS (2011) Statin myopathy: a common dilemma not reflected in clinical trials. *Cleve Clin J Med* **78**:393–403.
- Fisher CD, Lickteig AJ, Augustine LM, Oude Elferink RP, Besselsen DG, Erickson RP, and Cherrington NJ (2009) Experimental non-alcoholic fatty liver disease results in decreased hepatic uptake transporter expression and function in rats. *Eur J Pharmacol* **613**:119–127.
- Gandhi A, Moorthy B, and Ghose R (2012) Drug disposition in pathophysiological conditions. *Curr Drug Metab* **13**:1327–1344.
- Hardwick RN, Fisher CD, Canet MJ, Scheffer GL, and Cherrington NJ (2011) Variations in ATP-binding cassette transporter regulation during the progression of human nonalcoholic fatty liver disease. *Drug Metab Dispos* **39**:2395–2402.
- Hardwick RN, Fisher CD, Street SM, Canet MJ, and Cherrington NJ (2012) Molecular mechanism of altered ezetimibe disposition in nonalcoholic steatohepatitis. *Drug Metab Dispos* **40**:450–460.
- Ishigami M, Kawabata K, Takasaki W, Ikeda T, Komai T, Ito K, and Sugiyama Y (2001) Drug interaction between simvastatin and itraconazole in male and female rats. *Drug Metab Dispos* **29**:1068–1072.
- Iusuf D, Sparidans RW, van Esch A, Hobbs M, Kenworthy KE, van de Steeg E, Wagenaar E, Beijnen JH, and Schinkel AH (2012) Organic anion-transporting polypeptides 1a/1b control the hepatic uptake of pravastatin in mice. *Mol Pharm* **9**:2497–2504.
- Kalliokoski A and Niemi M (2009) Impact of OATP transporters on pharmacokinetics. *Br J Pharmacol* **158**:693–705.
- Lake AD, Novak P, Fisher CD, Jackson JP, Hardwick RN, Billheimer DD, Klimecki WT, and Cherrington NJ (2011) Analysis of global and absorption, distribution, metabolism, and elimination gene expression in the progressive stages of human nonalcoholic fatty liver disease. *Drug Metab Dispos* **39**:1954–1960.
- Lickteig AJ, Fisher CD, Augustine LM, Aleksunes LM, Besselsen DG, Slitt AL, Manautou JE, and Cherrington NJ (2007) Efflux transporter expression and acetaminophen metabolite excretion are altered in rodent models of nonalcoholic fatty liver disease. *Drug Metab Dispos* **35**:1970–1978.
- Link E, Parish S, Armitage J, Bowman L, Heath S, Matsuda F, Gut I, Lathrop M, and Collins R; SEARCH Collaborative Group (2008) SLCO1B1 variants and statin-induced myopathy—a genome-wide study. *N Engl J Med* **359**:789–799.
- McCullough AJ (2006) Pathophysiology of nonalcoholic steatohepatitis. *J Clin Gastroenterol* **40** (Suppl 1):S17–S29.
- Merrell MD and Cherrington NJ (2011) Drug metabolism alterations in nonalcoholic fatty liver disease. *Drug Metab Rev* **43**:317–334.
- Mofrad P, Contos MJ, Haque M, Sargeant C, Fisher RA, Luketic VA, Sterling RK, Shiffman ML, Stravitz RT, and Sanyal AJ (2003) Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. *Hepatology* **37**:1286–1292.
- Naik A, Belić A, Zanger UM, and Rozman D (2013) Molecular interactions between NAFLD and xenobiotic metabolism. *Front Genet* **4**:2.
- Nelson A, Torres DM, Morgan AE, Fincke C, and Harrison SA (2009) A pilot study using simvastatin in the treatment of nonalcoholic steatohepatitis: A randomized placebo-controlled trial. *J Clin Gastroenterol* **43**:990–994.
- Neuvonen PJ, Niemi M, and Backman JT (2006) Drug interactions with lipid-lowering drugs: mechanisms and clinical relevance. *Clin Pharmacol Ther* **80**:565–581.
- Niemi M (2010) Transporter pharmacogenetics and statin toxicity. *Clin Pharmacol Ther* **87**:130–133.
- Nseir W, Mograbi J, and Ghali M (2012) Lipid-lowering agents in nonalcoholic fatty liver disease and steatohepatitis: human studies. *Dig Dis Sci* **57**:1773–1781.
- Pasanen MK, Neuvonen M, Neuvonen PJ, and Niemi M (2006) SLCO1B1 polymorphism markedly affects the pharmacokinetics of simvastatin acid. *Pharmacogenet Genomics* **16**:873–879.
- Prueksaritanont T, Gorham LM, Ma B, Liu L, Yu X, Zhao JJ, Slaughter DE, Arison BH, and Vyas KP (1997) In vitro metabolism of simvastatin in humans [SBT] identification of metabolizing enzymes and effect of the drug on hepatic P450s. *Drug Metab Dispos* **25**:1191–1199.
- Prueksaritanont T, Qiu Y, Mu L, Michel K, Brunner J, Richards KM, and Lin JH (2005) Interconversion pharmacokinetics of simvastatin and its hydroxy acid in dogs: effects of gemfibrozil. *Pharm Res* **22**:1101–1109.
- Shitara Y and Sugiyama Y (2006) Pharmacokinetic and pharmacodynamic alterations of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors: drug-drug interactions and interindividual differences in transporter and metabolic enzyme functions. *Pharmacol Ther* **112**:71–105.
- Targher G, Bertolini L, Padovani R, Poli F, Scala L, Tessari R, Zenari L, and Falezza G (2006) Increased prevalence of cardiovascular disease in Type 2 diabetic patients with non-alcoholic fatty liver disease. *Diabet Med* **23**:403–409.
- Targher G, Bertolini L, Poli F, Rodella S, Scala L, Tessari R, Zenari L, and Falezza G (2005) Nonalcoholic fatty liver disease and risk of future cardiovascular events among type 2 diabetic patients. *Diabetes* **54**:3541–3546.
- Targher G, Bertolini L, Rodella S, Tessari R, Zenari L, Lippi G, and Arcaro G (2007) Nonalcoholic fatty liver disease is independently associated with an increased incidence of cardiovascular events in type 2 diabetic patients. *Diabetes Care* **30**:2119–2121.
- Thompson PD, Clarkson PM, and Rosenson RS; National Lipid Association Statin Safety Task Force Muscle Safety Expert Panel (2006) An assessment of statin safety by muscle experts. *Am J Cardiol* **97** (8A):69C–76C.
- Verbeeck RK (2008) Pharmacokinetics and dosage adjustment in patients with hepatic dysfunction. *Eur J Clin Pharmacol* **64**:1147–1161.
- Voorra D, Shah SH, Spasojevic I, Ali S, Reed CR, Salisbury BA, and Ginsburg GS (2009) The SLCO1B1*5 genetic variant is associated with statin-induced side effects. *J Am Coll Cardiol* **54**:1609–1616.
- Watson RG and McLeod HL (2011) Pharmacogenomic contribution to drug response. *Cancer J* **17**:80–88.
- Wilke RA, Ramsey LB, Johnson SG, Maxwell WD, McLeod HL, Voorra D, Krauss RM, Roden DM, Feng Q, and Cooper-Dehoff RM, et al.; Clinical Pharmacogenomics Implementation Consortium (CPIC) (2012) The clinical pharmacogenomics implementation consortium: CPIC guideline for SLCO1B1 and simvastatin-induced myopathy. *Clin Pharmacol Ther* **92**:112–117.
- Yang AY, Sun L, Musson DG, and Zhao JJ (2005) Application of a novel ultra-low elution volume 96-well solid-phase extraction method to the LC/MS/MS determination of simvastatin and simvastatin acid in human plasma. *J Pharm Biomed Anal* **38**:521–527.
- Zaher H, Meyer zu Schwabedissen HE, Tirona RG, Cox ML, Obert LA, Agrawal N, Palandra J, Stock JL, Kim RB, and Ware JA (2008) Targeted disruption of murine organic anion-transporting polypeptide 1b2 (Oatp1b2/Slco1b2) significantly alters disposition of prototypical drug substrates pravastatin and rifampin. *Mol Pharmacol* **74**:320–329.

Address correspondence to: Nathan J. Cherrington, Department of Pharmacology and Toxicology, University of Arizona, Tucson, 1703 E. Mabel Street, Tucson, AZ 85721. E-mail: cherrington@pharmacy.arizona.edu