Research Article

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Synergistic interaction between genetics and disease on pravastatin disposition

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Background & Aims: A genome wide association study and multiple pharmacogenetic studies have implicated the hepatic uptake transporter organic anion transporting polypeptide-1B1 (OATP1B1) in the pharmacokinetics and musculoskeletal toxicity of statin drugs. Other OATP uptake transporters can participate in the transport of pravastatin, partially compensating for the loss of OATP1B1 in patients carrying the polymorphism. Non-alcoholic steatohepatitis (NASH) in humans and in a diet-induced rodent model alter the expression of multiple OATP transporters.

Methods: To determine how genetic alteration in one Oatp transporter can interact with NASH-associated changes in Oatp expression we measured the disposition of intravenously administered pravastatin in Slco1b2 knockout (*Slco1b2*$^{-/-}$) and wild-type (WT) mice fed either a control or a methionine and choline deficient (MCD) diet to induce NASH.

Results: Genetic loss of *Oatp1b2*, the rodent ortholog of human OATP1B transporters, caused a modest increase in pravastatin plasma concentrations in mice with healthy livers. Although a diet-induced model of NASH decreased the expression of multiple hepatic Oatp transporters, it did not alter the disposition of pravastatin compared to WT control mice. In contrast, the combination of NASH-associated decrease in compensatory Oatp transporters and Oatp1b2 genetic loss caused a synergistic increase in plasma area under the curve (AUC) and tissue concentrations in kidney and muscle.

Conclusions: Our data show that NASH alters the expression of multiple hepatic uptake transporters which, due to overlapping substrate specificity among the OATP transporters, may combine with the pharmacogenetic loss of OATP1B1 to increase the risk of statin-induced adverse drug reactions.

Keywords: Non-alcoholic steatohepatitis; Statin; Myopathy; Adverse drug reaction.

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Abbreviations: ADR, adverse drug reaction; Oatp, organic anion transporting polypeptide; SNP, single nucleotide polymorphism; NASH, Non-alcoholic steatohepatitis; NAFLD, Non-alcoholic fatty liver disease; MCD, methionine and choline deficient; AUC, area under the curve; Mrp, multidrug resistance-associated protein.

Introduction

Multiple biological factors can impact drug disposition and the occurrence of adverse drug reactions (ADRs), including pharmacogenetic variation in transporters and liver diseases [1–3]. Single nucleotide polymorphisms (SNPs) in *SLCO1B1*, the gene encoding human hepatic organic anion transporting polypeptide-1B1 (OATP1B1), have been shown to cause altered transporter function and altered disposition of multiple statin drugs, including pravastatin [2,4,5]. Statin drugs are among the most widely prescribed drugs worldwide and are primarily used to reduce hyperlipidemia and the risk of heart disease and stroke [6,7]. Although statins are relatively safe with few side effects, it is believed that these drugs are underutilized, partially due to the occurrence of statin-induced muscle toxicities [6,7]. The Prediction of Muscular Risk in Observational conditions (PRIMO) study showed that muscular symptoms were reported in 5.1–18.2% of patients taking high-dosage statins, with 10.9% of patients taking pravastatin reporting symptoms of muscle toxicity [6]. Importantly, statin-induced muscle toxicities are dose-dependent and are related to plasma drug concentrations [4,7–10]. Pharmacogenetic variation in statin disposition has gained considerable attention as a potential risk factor for statin-induced muscle toxicities and recent studies have linked SNPs in *SLCO1B1* to increased statin plasma concentrations and statin-induced muscle toxicities [9–11]. In addition to pharmacogenetic variation there are other variables, such as disease-specific alterations in drug transporters that can influence drug disposition and occurrence of ADRs.

It is well recognized that liver diseases can alter drug disposition and require dose adjustment to maintain drug concentrations within the therapeutic window [1,12]. Our group has shown that non-alcoholic steatohepatitis (NASH), a progressive

form of non-alcoholic fatty liver disease (NAFLD), causes altered drug transporter function and contributes to altered drug disposition [3,13–16]. We have also shown that diet-induced NASH in rodents decreases the expression of multiple hepatic Oatp uptake transporters and increases the plasma concentrations of sulfobromophthalein [15]. There is a high degree of overlap in substrate specificity between the various OATP isoforms, and the coordinated down-regulation of numerous uptake transporters in NASH may reduce the potential for compensatory transport. We hypothesized that the combination of genetic- and disease-specific changes in drug transporters will synergistically alter the disposition of the OATP substrate pravastatin.

We utilized a genetic knockout model of *Oatp1b2*, the primary rodent uptake transporter for pravastatin, and a methionine and choline deficient (MCD) diet-induced model of NASH to test the impact of genetics (*Oatp1b2* knockout), disease (NASH), and the combined gene-by-environment effect on pravastatin disposition.

Materials and methods

Animals

Male C57Bl6 mice from Jackson Laboratory (Bar Harbor, ME) and *Slco1b2*$^{-/-}$ mice from Dr. Curtis Klaassen (University of Kansas Medical Center) at 5 months of age were housed on a 12 h light and 12 h dark cycle in the University of Arizona animal care facility. Mice were provided either a methionine and choline deficient (MCD) diet or a control diet replete with methionine and choline (control) from Dyets Inc. (Bethlehem, PA) *ad libitum* for six weeks. Animals were anesthetized and surgery performed to place cannulas into the carotid artery and jugular vein for pravastatin disposition studies as previously described [17]. Pravastatin (Sigma Aldrich, St. Louis, MO) was solubilized in sterile normal saline, and administered into the jugular vein (10 mg/kg, 5 ml/kg). Blood was collected from the carotid artery 5, 10, 25, and 40 min after pravastatin administration into heparinized tubes and plasma was isolated by centrifugation. After the 40 min blood collection the mice were euthanized and liver, kidney, gastrocnemius, and soleus tissues were collected. A slice of liver and kidney tissues were fixed in formalin and embedded in paraffin for histological analysis. Tissues were stained with hematoxylin and eosin and scored by a trained veterinary pathologist for lipid accumulation, necrosis, inflammation, fibrosis, and biliary hyperplasia. Pathology scores were as follows: 0, no significant lesions (0%); 1, minimal (<10%); 2, mild (10–25%); 3, moderate (25–40%); 4, marked (40–50%); 5, severe (>50%). The remaining tissues were snap frozen for mRNA or protein isolation or pravastatin quantification. The animal studies were approved by the University of Arizona Animal Care and Use Committee.

Human liver samples

Human liver tissue was acquired from the National Institutes of Health-funded Liver Tissue Cell Distribution System (LTCDS) which was funded by NIH Contract #N01-DK-7-0004/HHSN267200700004C. Clinical and demographic information of these human liver samples has been described previously [18]. Tissues were collected postmortem and preserved as either frozen or paraffin embedded tissue. The samples were diagnosed as normal, steatotic, and NASH by a Liver Tissue Cell Distribution System medical pathologist according to the NAFLD activity scoring categorization [19].

mRNA analysis

Total RNA was isolated from mouse livers using RNAzol B reagent from Tel-Test Inc. (Friendswood, TX) according to the manufacturer's protocol. The branched DNA (bDNA) assay was used to quantify mRNA transcripts using gene specific oligonucleotide probes for *Oatp1a1*, *Oatp1a4*, *Oatp1b2*, *Oatp2b1*, *Mrp2*, *Mrp3*, *Mrp4*, *OATP1B1*, and *OATP1B3*. A QuantiGene 1.0 Discovery Assay Kit from Affymetrix Inc. (Santa Clara, CA) was used according to the manufacturer's protocol and as previously described [20]. Luminescence was measured using a Quantiplex 320 bDNA luminometer interfaced with Quantiplex Data Management Software (version 5.02). *OATP2B1* mRNA expression was quantified from a previously validated microarray dataset [13].

Western blot analysis

Whole cell protein lysates were prepared from mouse livers as previously described [16]. Portions of the whole cell lysates were subjected to ultracentrifugation at 100,000 xg for one h to collect a membrane enriched fraction. Sixty micrograms of whole cell lysate or 30 μg of membrane preparation were separated on 7.5% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The following antibodies were used for each protein: Oatp1a1, Santa Cruz Biotechnology (Santa Cruz, CA) sc-47265; Oatp1a4, sc-18436; Oatp1b2, sc-47270; Mrp2, M_2III-5 clone Kamiya Biomedical Company (Seattle, WA) MC-267; Mrp3, sc-5775; Mrp4, M_4I-10 clone generated by George L. Scheffer (Amsterdam, The Netherlands); OATP1B1, Progen Biotech (Heidelberg, Germany); OATP1B3, gift from Dr. Bruno Hagenbuch (University of Kansas Medical Center); Oatp2b1/OATP2B1 sc-135099. Relative protein levels were measured using Image J software from the National Institutes of Health (Bethesda, MD) and each protein was normalized to either Erk-2 (sc-154) or pan-cadherin (Abcam, Ab16505).

Pravastatin quantification

The methods for pravastatin quantification in mouse plasma, liver, kidney, gastrocnemius, soleus, and urine were adapted from previously published methods [21]. Pravastatin (catalog #P4498) and 3-α-isopravastatin (catalog #H952310) were purchased from Sigma Aldrich and Toronto Research Chemicals (Ontario, Canada), respectively. A deuterium labeled internal standard pravastatin-d3 (catalog #P702002) was purchased from Toronto Research Chemicals. A Waters (Milford, MA) Micromass Quattro Premier XE tandem mass spectrometer coupled to an Acquity UPLC was used in the Arizona Laboratory for Emerging Contaminants at the University of Arizona. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) and was pumped at a flow rate of 0.3 ml/min through a Waters Acquity UPLC BEH C19 column (1.7 μm, 2.1 × 50 mm). The UPLC gradient started at 20% B and increased to 36% B over four min, then was equilibrated to 20% B for one min before the next injection. Multiple reaction monitoring in negative mode was used to detect pravastatin at *m/z* 423.3 >303.3, 3-α-isopravastatin at *m/z* 423.3 >303.3, and pravastatin-d3 at *m/z* 426.3 >303.3. Liver, kidney, gastrocnemius, and soleus tissues were homogenized by grinding the tissue in liquid nitrogen (~100 mg for liver, kidney, and gastrocnemius, and ~10 mg for soleus), followed by addition of 4% bovine serum albumin. The samples were vortexed vigorously, frozen, thawed, and then centrifuged at 13,000 rpm for 10 min. Twenty-microliters of either plasma or tissue homogenate or 30 μl of urine was mixed with 30 μl of acetonitrile that contained pravastatin-d3, vortexed, and centrifuged at 13,000 rpm for 10 min. Forty-microliters of the supernatant was mixed with 80 μl of water and 10 μl was injected onto the BEH column.

Statistical analyses

All results are represented as mean ± standard error of the mean (SEM). For all comparisons within the rodent studies Two-way ANOVA statistical analyses were employed with a Bonferroni multiple comparisons post-test to compare between control and NASH animals within each genotype. For the human OATP expression data, One-way ANOVA was employed with Dunnett's multiple comparisons post-test to compare the Steatosis and NASH samples to control samples.

Results

Methionine and choline deficient diet induces NASH in wild-type and Slco1b2$^{-/-}$ *mice*

The MCD diet is a common model used to study NASH because it recapitulates many of the characteristic liver pathology features and expression patterns in drug metabolizing enzymes and transporters observed in the human condition [22]. Although this