ALTERED UDP-GLUCURONOSYLTRANSFERASE EXPRESSION IN NONALCOHOLIC STEATOHEPATITIS AND IMPLICATIONS FOR DRUG METABOLISM

By

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Abstract

Nonalcoholic steatohepatitis (NASH) affects the regulation of drug metabolizing enzymes and transporters, which leads to a variability in the disposition of drugs for individuals. Previous studies have shown that UDP-glucuronosyltransferase (UGT) activity and expression can change over the progression of nonalcoholic fatty liver disease (NAFLD). This study examined the enzyme activity of the UGT1a9 isoform and the UGT2b cluster in NASH mice models. There was a significant upregulation in the protein expression of UGT1a9 and UGT2b, while the mRNA expression of UGT1a9 decreased significantly in the NASH mice. This data suggests that, while there may be posttranscriptional processing responsible for the contrast in mRNA and protein expression, NASH may alter the glucuronidation of compounds, especially therapeutic drugs. Future study should focus on how UGT regulation is changed in NASH patients to better predict drug exposure and adverse drug events.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common type of chronic liver disease that presents with ≥ 5% hepatic steatosis, in the absence of other liver diseases, chronic hepatitis, significant alcohol consumption, or use of medications that lead to steatosis (Younossi et al., 2016). The prevalence of the disease is about 25% worldwide, and has associated metabolic comorbidities with obesity, diabetes mellitus, hyperlipidemia, hypertension, and metabolic syndrome (Younossi et al., 2016). Nonalcoholic steatohepatitis (NASH), is a more progressive type of NAFLD that presents in a smaller subset of patients. NASH is diagnosed via liver biopsy and lesions most accepted for representing the disease include steatosis, hepatocyte degeneration, lobular necroinflammation, and hepatic fibrosis (Brunt, 2001). There remains some controversy over the mortality of NASH, which could be associated with a significant presence of fibrosis; however, it is commonly accepted that most of the progression of fibrosis occurs in patients with NASH (Younossi et al., 2016). The disease progression of NAFLD ranges from simple hepatic steatosis to NASH, and could progress into cirrhosis and hepatocellular carcinoma (Li et al., 2017).

Studies have shown that during this progression of NAFLD and, more specifically, NASH, there is an alteration in gene expression in drug metabolizing enzymes and transporters that take drugs in and out of the liver (Lake et al., 2011). Drug metabolism is important because it provides a way for drugs to enter the body, be used, and then excreted. Metabolism occurs mainly in the liver and intestines in either sequential or competing processes including Phase I reactions, such as oxidation, reduction, and hydrolysis, and Phase II reactions, such as glucuronidation, sulfation, acetylation, and methylation (Wilkinson, 2005). This metabolism, and the rate at which it occurs, often determines the extent of drug exposure for a patient before the

drug is excreted from the body. If there is too little exposure to a drug, then the treatment is ineffective, while if there is too much exposure to a drug, adverse drug events can occur (Wilkinson, 2005).

An important family of enzymes involved in drug metabolism are uridine diphosphate (UDP)-glucuronosyltransferases (UGTs). These enzymes catalyze the glucuronidation of a wide-range of lipophilic chemicals and play a major role in the excretion of compounds, which allows for detoxification (Meech & Mackenzie, 1997). As a phase II reaction, the conjugation of the glucuronide to the substrate creates a more hydrophilic metabolite with a higher molecular weight, and in many instances allows for easier excretion into the bile and urine from the hepatocyte (Jancova, Anzenbacher, & Anzenbacherova, 2010; Meech & Mackenzie, 1997). UGTs are found primarily in the liver of mammals, specifically on the endoplasmic reticulum, and small hydrophobic molecules that lack carbohydrates, known as aglycones, are typical substrates for glucuronidation (Meech & Mackenzie, 1997). Therapeutic drugs, along with other endogenous and exogenous compounds, such as bilirubin and steroid hormones, can be glucuronidated.

Understanding how drugs are metabolized through these phase II reactions is important because many of the enzymes that catalyze those reactions play an important role in the metabolic inactivation of xenobiotics (Jancova et al., 2010). While there are some exceptions, such as morphine glucuronide metabolites being more active than the unconjugated drug or acyl glucuronides producing metabolites with a significant risk for toxicity, most glucuronides follow the pattern of inactivation and clearance (Hardwick et al., 2013; Meech & Mackenzie, 1997). Either way, as patients are observed to have differing glucuronidation rates, the rates of drug metabolism also differ. This is important considering the difference that NASH can have on gene

expression in drug metabolizing enzymes. The altered expression, which then alters metabolism rate in drugs where the exact drug exposure could be the difference between treatment and toxicity, opens up the possibility for a greater number of adverse drug events in patients with NASH.

Adverse drug events (ADEs) are unwanted and usually harmful or unpleasant outcomes that are unintended and associated with the administration of a drug following normal doses for prophylaxis, diagnosis, and treatment of disease (Edwards & Aronson, 2000). These can include toxic effects, like digoxin toxicity, and side effects like the anticholinergic effect of antidepressants. ADEs such as these are usually dose-related, common, predictable and manageable (Edwards & Aronson, 2000). There are other reactions, however, such as myocardial ischemia, carcinogenesis, and even death that classify as more serious ADEs. The causes for these are numerous, including incorrect dosing, an allergy to a drug, altered drug metabolizing or transport enzymes and the contraindications that can occur between disease-drug interactions and drug-drug interactions (Edwards & Aronson, 2000).

Studies have shown that NASH can have an effect on the disposition of different drugs, such as pravastatin and morphine (Clarke et al., 2014; Dzierlenga et al., 2015). Previous studies have also looked at the expression of different UGT isoforms to identify changes in activity and expression during the different stages of NAFLD (Hardwick et al., 2013). The purpose of this study, however, was to look at the specific isoform UGT1a9 and the UGT2b cluster to identify if an alteration of protein expression existed between control and NASH models. From these results, implications could be drawn for drugs that use these isoforms for phase II metabolism, in which NASH may affect a patient's drug exposure and adverse drug events can be better predicted and managed.

Materials and Methods

Animals

The liver tissue samples used in this study were obtained previously using male C57/BL6 mice from Jackson Laboratory, *Slco1b2*$^{-/+}$ *and Slco1b2*$^{-/-}$, from Dr. Ivan Csanaky (The University of Kansas Medical Center). They were housed at The University of Kansas Medical Center animal care facility on a 12-hour light and 12-hour dark cycle. The mice in the control group were fed a methionine and choline sufficient diet, while the mice in the methionine- and choline-deficient (MCD) group were fed a MCD diet for six weeks to induce NASH.

RNA Isolation and Quantitative Reverse Transcription-PCR

RNA was isolated from the liver tissues using the RNAzol B reagent (Tel-Test, Friendswood, TX) and the manufacturer's protocol. cDNA was prepared using the ReadyScript® cDNA synthesis kit (Sigma-Aldrich, St. Louis, MO). Each reaction well contained 1x KiCqStart™ SYBR® green master mix, 1 μL of forward and reverse primers, 2 μL of cDNA template and diethyl pyrocarbonate (DEPC) water, up to 20 μL, run in duplicate. The following forward and reverse primers were obtained from Sigma-Aldrich: UGT1a9, forward and reverse, and beta-Actin, forward and reverse. qRT-PCR was run on an ABI StepOnePlus Real Time PCR system with the standard SYBR® green PCR cycling profile. The mRNA data was normalized to beta-Actin and the fold change was determined using the delta-delta C_T method.

Protein Preparation

Three hundred milligrams of liver tissue were homogenized in NP40 buffer with one protease inhibitor cocktail tablet (Roche, Indianapolis, IN) per 25 mL. The homogenized tissues were then incubated for 2 hours at 4 °C and then centrifuged for 30 minutes at 10,000xg. The supernatant was transferred into one milliliter microcentrifuge tubes and stored at -80 °C. Protein

concentrations of the supernatant were quantified by the Pierce BCA Protein Quantification Assay Kit (Thermo Fisher Scientific, Waltham, MA) following standard protocol.

Immunoblot Analysis

One hundred micrograms of whole cell lysates were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked in a solution of 5% nonfat milk product in Tris-buffered saline/Tween-20 (TBST) for at least one hour and incubated in primary antibody overnight at 4 °C. The following antibodies were used to detect proteins: Erk2 (Santa Cruz Biotechnology, Dallas, TX, sc-125), UGT2b (sc-271777), and UGT1a9 (Abnova, Taipei, Taiwan, H00054600-B01P). Densitometry of the proteins was determined using ImageJ software (National Institutes of Health, Bethesda, MD) and relative expression was normalized to the housekeeping protein Erk2 (sc-125).

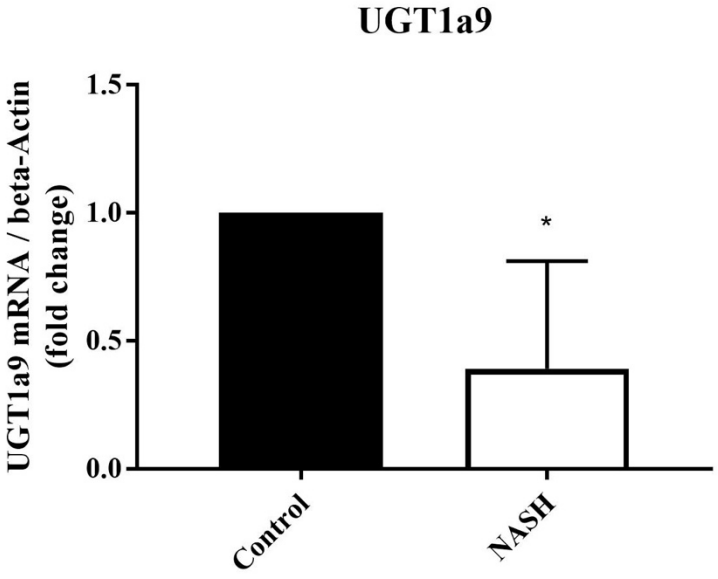
Statistics

All results are represented as the mean ± the standard deviation. Parametric unpaired t-tests were used as statistical analyses to compare between the control and NASH animal groups.

Results

Hepatic UGT1a9 mRNA expression altered in NASH mice models

The mRNA expression of UGT1a9 was normalized to the mRNA expression of beta-Actin, and the fold change is shown in Figure 1. The UGT1a9 mRNA expression was significantly decreased in the NASH mice model compared to the control mice model, with a P value of < 0.0001.



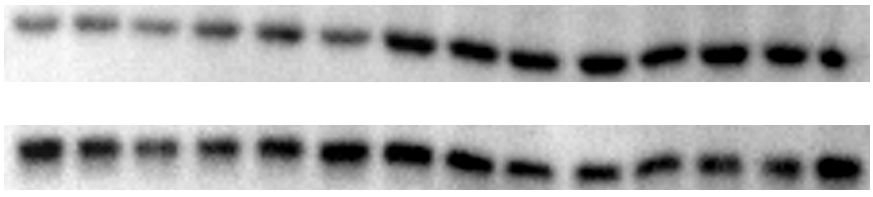
Unpaired t test	UGT1a9
P value	< 0.0001

Figure 1. Hepatic mRNA expression of UGT1a9 in control and MCD (NASH) mice. Hepatic mRNA expression was quantified and normalized to beta-Actin. Data represents the mean ± SD. Control n=14, NASH n=14. Parametric unpaired t-test was performed to determine statistical significance and * indicates a significant difference between control and NASH mice.

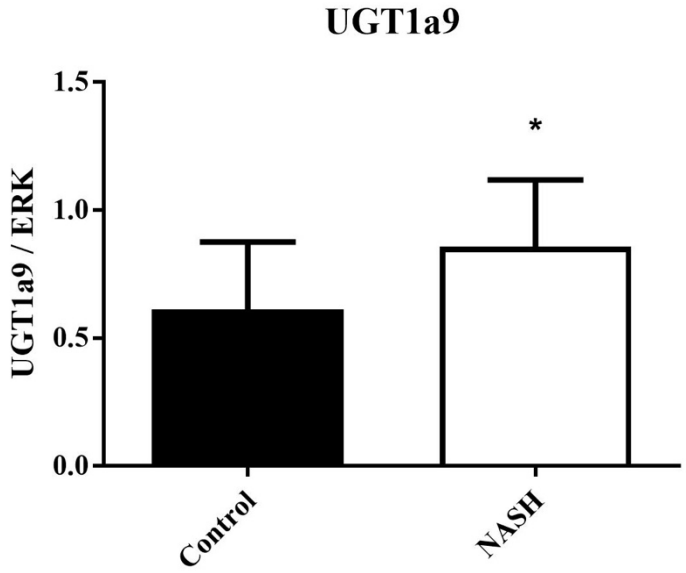
Hepatic UGT protein expression alteration in NASH mice models

Western blot analysis was performed on control and NASH mice to determine the relative protein expression of UGT1a9 (Figure 2A) and the UGT2b cluster (Figure 3A). The fold change of the protein expression was normalized to the ERK2 protein expression in both UGT1a9 (Figure 2B) and UGT2b (Figure 3B). Both the UGT1a9 and UGT2b expressions were significantly increased in the NASH mice when compared to the control mice, with a P value of 0.0047 for UGT1a9 and 0.0053 for UGT2b.

A



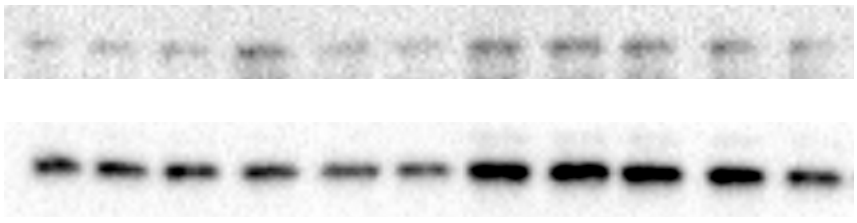
B



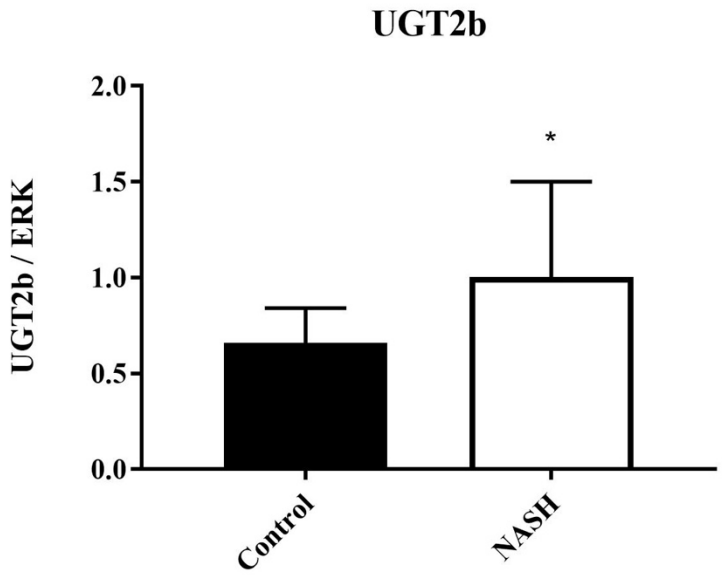
Unpaired t test	UGT1a9
P value	0.0047

Figure 2. Hepatic protein expression of UGT1a9 in control and MCD (NASH) mice. Hepatic protein expression was measured by Western blot analysis (A). A Densitometry analysis was performed of the Western blot and the protein expression relative to ERK was shown (B). Data represents the mean ± SD. Control n=21, NASH n=20. Parametric unpaired t-test was performed to determine statistical significance and * indicates a significant difference between control and NASH mice.

A



B



Unpaired t test	UGT2b
P value	0.0053

Figure 3. Hepatic protein expression of UGT2b in control and MCD (NASH) mice. Hepatic protein expression was measured by Western blot analysis (A). A Densitometry analysis was performed of the Western blot and the protein expression relative to ERK was shown (B). Data represents the mean ± SD. Control n=21, NASH n=20. Parametric unpaired t-test was performed to determine statistical significance and * indicates a significant difference between control and NASH mice.

Discussion

While the mice models cannot completely mimic the population of NASH human patients, they provide a way to predict some metabolic changes that may occur with NASH. Significant changes in the mRNA expression did not correspond to the increased protein expression of the UGT1a9 isoform. This indicates that there could have been possible posttranscriptional processing, such as posttranslational modification that caused the protein to not be degraded or mRNA splicing, which has been identified in previous studies for differences between mRNA and protein expression (Court, 2010; Hardwick et al., 2013).

With these differences, though, the importance of the significantly increased protein expression of both the UGT1a9 isoform and the UGT2b cluster cannot be discounted. This protein expression implies the possibility of increased glucuronidation, which can work in three ways: (1) there may be increased activation of the active metabolites of therapeutic drugs, (2) there may be increased inactivation of the active metabolites of therapeutic drugs, and (3) there may be increased acyl glucuronides, which leads to a higher risk of toxicity (Meech & Mackenzie, 1997). These all lead to the possibility of an increased or decreased blood drug concentration in patients with NASH when receiving a standard dosing (Fujiwara, Yoda, & Tukey, 2018). Previous studies have shown a variation in drug disposition when transporters and other metabolizing enzymes are affected by NASH (Clarke et al., 2014; Dzierlenga et al., 2015; Li et al., 2017). Studies have also shown variation between individuals for drug biotransformation when there are genetic polymorphisms in UGTs (Radominska-Pandya, Czernik, Little, Battaglia, & Mackenzie, 1999). However, there have not been many studies that focus solely on how glucuronidation is affected by NASH, which is where this data can make

implications for future studies of drugs that undergo glucuronidation as a major part of their metabolism.

Out of the clinically used drugs available, more than 20% undergo glucuronidation (Fujiwara et al., 2018). Tramadol, which is a centrally acting opioid analgesic, is used to treat pain and is rapidly absorbed when taken by oral administration (Gong, Stamer, Tzvetkov, Altman, & Klein, 2014). It is metabolized mainly through O- and N-demethylation, and the O-demethylation forms the main analgesic metabolite, O-desmethyltramadol (M1). This active metabolite, M1, undergoes glucuronidation during phase II metabolism, which inactivates it mostly through UGT2b7 and UGT1a8 (Gong et al., 2014). Assays have shown that almost 24% of the administered dose of tramadol is excreted as O-desmethyltramadol glucuronide, which indicates a high glucuronidation rate of the active metabolite (Gong et al., 2014). Since our results show that the UGT2b cluster has a significant upregulation in protein expression in NASH mice models, it can be implied that the glucuronidation of M1 may increase or occur at a faster rate, which would alter the analgesic effects of tramadol for a patient with NASH.

Another drug that undergoes glucuronidation is ibuprofen, which is a non-steroidal anti-inflammatory commonly used to treat pain and inflammation. The two major metabolites of ibuprofen, carboxy-ibuprofen and 2-hydroxy-ibuprofen are glucuronidated and account for about 37% and 25% of the excreted dose through urine, respectively (Mazaleuskaya et al., 2015). This phase II metabolism, with a combined over 60% clearance of active metabolites, has been shown to be performed by UGT1a3, UGT1a9, UGT2b4, UGT2b7, and UGT2b17 (Mazaleuskaya et al., 2015). As both UGT1a9 and the UGT2b were shown to have an increased protein expression in NASH, the implication can be drawn that, like tramadol, there may be increased or faster glucuronidation, which would result in alteration of the effects of ibuprofen because of greater

drug clearance from the body. There is also about 10-15% of an ibuprofen dose that is glucuronidated directly to ibuprofen-acyl glucuronides (Mazaleuskaya et al.). While studies have shown that the ibuprofen-acyl glucuronides do not lead to high liver toxicity, the increased glucuronidation in NASH also provides an interesting question of whether that toxicity will change in patients with NASH (Mazaleuskaya et al.).

Finally, the glucuronidation of sorafenib is involved in about 19% of the dose's clearance from a patient's body through urine (Gong et al., 2017). As an oral anticancer drug, sorafenib's drug disposition is of great concern. Primary metabolism of sorafenib goes through two pathways in the liver: phase I oxidation by cytochrome P450 3A4 and phase II glucuronidation by UGT1a9 (Gong et al., 2017). This high reliance upon UGT1a9 for metabolism, with the data that shows an upregulation in protein expression of UGT1a9 in NASH models, indicates that there may be altered disposition of sorafenib in NASH patients.

Conclusion

The NASH mice models served as profiles for the mRNA expression of UGT1a9 and protein expression of UGT1a9 and the UGT2b cluster, when compared to controls. While, this may not be completely reflective of alterations in human NASH, these models can be carefully considered when studying how drug disposition can change due to the disease. Future study is encouraged to determine how well these models apply to human NASH patient metabolism. Extrapolation of this data can also be used for future studies to focus on how alterations in UDP-glucuronosyltransferase expression affects the disposition of drugs that are glucuronidated in the process of activating or clearing metabolites from the body. Overall, safety against adverse drug events could come from a greater understanding into how NASH in humans affects UGT regulation.

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