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Mechanism of Altered Metformin Distribution in Nonalcoholic Steatohepatitis

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Metformin is an antihyperglycemic drug that is widely prescribed for type 2 diabetes mellitus and is currently being investigated for the treatment of nonalcoholic steatohepatitis (NASH). NASH is known to alter hepatic membrane transporter expression and drug disposition similarly in humans and rodent models of NASH. Metformin is almost exclusively eliminated through the kidney primarily through active secretion mediated by Oct1, Oct2, and Mate1. The purpose of this study was to determine how NASH affects kidney transporter expression and metformin pharmacokinetics. A single oral dose of [¹⁴C]metformin was administered to C57BL/6J (wild type [WT]) and diabetic *ob/ob* mice fed either a control diet or a methionine- and choline-deficient (MCD) diet. Metformin plasma concentrations were slightly increased in the WT/MCD and *ob*/control groups, whereas plasma concentrations were 4.8-fold higher in *ob*/MCD mice compared with WT/control. The MCD diet significantly increased plasma half-life and mean residence time and correspondingly decreased oral clearance in both genotypes. These changes in disposition were caused by *ob/ob*- and MCD diet-specific decreases in the kidney mRNA expression of Oct2 and Mate1, whereas Oct1 mRNA expression was only decreased in *ob*/MCD mice. These results indicate that the diabetic *ob/ob* genotype and the MCD disease model alter kidney transporter expression and alter the pharmacokinetics of metformin, potentially increasing the risk of drug toxicity.

Metformin is recognized as a first-line antidiabetic drug used to treat type 2 diabetes mellitus (T2DM) that belongs to the biguanide class of drugs. In addition to

its antidiabetic effects, metformin has also been reported to provide antiobesity, antihyperglycemic, antilipidemic, antineoplastic, and cardioprotective benefits to patients (1). These facts have made metformin one of the most prescribed drugs, with millions of prescriptions dispensed each year in the U.S. (1). Lactic acidosis is a severe, life-threatening, and dose-dependent adverse effect of metformin characterized by low arterial pH that has an incidence of approximately three cases per 100,000 patients per year (2). Lactic acidosis is caused by accumulation of metformin within the patient during the course of treatment and is known to be fatal in ~50% of patients. Metformin is contraindicated in patients with renal disease or renal dysfunction because it is almost exclusively eliminated through the kidneys into the urine unchanged (3). Renal elimination of metformin is carried out primarily by active secretion, with total renal clearance reported to be five times higher than the glomerular filtration rate (4). In vitro and in vivo studies have implicated renal organic cation transporter 1 (Oct1) and Oct2 on the basolateral membrane of proximal tubule cells and multidrug and toxin extrusion 1 (Mate1) on the apical membrane as the major transporters involved in active secretion of metformin (2,5–7). Given the fact that metformin is dependent on renal elimination, there is a need to investigate other potential sources of variability in kidney function that may impact metformin disposition and the occurrence of adverse drug reactions.

There is a growing body of evidence to indicate that nonalcoholic fatty liver disease (NAFLD) causes extrahepatic changes in the function of organs such as the kidney. NAFLD is now the most common liver disease in

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the U.S. and the most severe form of the disease, nonalcoholic steatohepatitis (NASH), is estimated to be present in 5.7–17% of the adult U.S. population (8). T2DM is highly associated with NASH, with >60% of patients with NASH also reported to have T2DM, and patients with T2DM are at the greatest risk for progression of NAFLD to NASH (9). Recent research has shown that NAFLD and NASH are associated with the occurrence of chronic kidney disease (CKD), independent of other risk factors such as age, sex, BMI, hypertension, diabetes, smoking, and hyperlipidemia (10–18). Lower glomerular filtration rate and a greater frequency of CKD has been reported in NASH patients compared with control patients matched for age, sex, and BMI (17,18). Currently it is not known what effect NAFLD has on the expression and function of transporters within the kidney and how this may impact elimination of drugs such as metformin. These data suggest that NASH patients may be an at-risk population for altered pharmacokinetics of renally excreted drugs.

In the current study, the hepatic and extrahepatic effects of liver disease on metformin pharmacokinetics were determined by modeling NASH with the methionine- and choline-deficient (MCD) diet and the *ob/ob* diabetic mouse strain.

RESEARCH DESIGN AND METHODS

Animal Study

C57BL/6J (wild type [WT]) and leptin-deficient (*ob/ob*) male mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age ($n = 5$ for each group) and housed on a 12-h light and 12-h dark cycle in The University of Arizona animal care facility. The animal studies were approved by The University of Arizona Animal Care and Use Committee. Mice were provided either an MCD diet or a control diet replete with methionine and choline (control) from Dyets Inc. (Bethlehem, PA) ad libitum. WT mice and *ob/ob* mice were fed the diets for 6 and 4 weeks, respectively. Mice were fasted for 12 h prior to receiving a single oral dose (10 mL/kg) of [14 C]metformin (50 mg/kg and 100 μ Ci/kg). After dosing, mice were housed in metabolic cages by treatment group for 12 h in order to collect urine and feces. Blood was collected at 0.5, 1, 2, 4, 6, 8, and 12 h into heparinized tubes and subjected to centrifugation at 10,000 rpm for 1 min to isolate plasma. Approximately 50 mg of liver, kidney, and muscle tissue were collected and processed with 0.5 mL of Solvable (PerkinElmer, Waltham, MA) for 3.5 h at 60°C, followed by the addition of 5 mL of Ultima Gold (PerkinElmer). Radioactivity in plasma, liver, kidney, muscle, urine, and feces was measured by liquid scintillation counting. The pharmacokinetics of metformin was determined via noncompartmental analysis using PKSolver (19). Pharmacokinetics end points include plasma area under the curve (AUC), plasma half-life, plasma mean residence time, oral volume of distribution, and oral clearance.

Histopathological Analysis and Plasma Chemistries

Formalin-fixed and paraffin-embedded liver and kidney tissues were stained with hematoxylin-eosin and scored by a board-certified veterinary pathologist. Liver tissues were scored for lipid accumulation, necrosis, inflammation, fibrosis, and biliary hyperplasia, and kidney tissues were scored for necrosis, tubule degeneration and regeneration, tubule dilation, tubule casts, and nephropathy. 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%); and 5, severe (>50%). The remaining tissues were snap frozen for mRNA isolation and quantification. The terminal plasma collection was used to quantify insulin and glucose. The dose of metformin was low enough and the plasma collection long enough after the dose that metformin likely did not have an effect on blood glucose levels (20). Insulin was measured using an ELISA kit (Millipore, Billerica, MA) and glucose was measured using an absorbance-based assay kit (Abcam, Cambridge, MA), both according to the manufacturer's protocol.

mRNA Analysis

Total RNA was isolated from mouse livers and kidneys using RNAzol B reagent from Tel-Test Inc. (Friendswood, TX) according to the manufacturer's protocol. The branched DNA assay was used to quantify mRNA transcripts using gene-specific oligonucleotide probes for Oct1, Oct2, and Mate1 (Supplementary Table 1). Total RNA (1 μ g/ μ L; 10 μ L per well) was added to each well of a 96-well plate containing 50 μ L of the appropriate probe set. 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 (21). Luminescence was measured using a Quantiplex 320 branched DNA luminometer interfaced with Quantiplex Data Management Software (version 5.02).

Statistical Analysis

All results are represented as mean \pm SEM. For all comparisons within the rodent studies, two-way ANOVA statistical analyses were used with a Bonferroni multiple comparisons posttest to compare between control and MCD mice within each genotype. Histopathological data were rank ordered before statistical analysis. An outlier test using a modified z score threshold of 3.5 was performed to identify potential outliers, and it was determined that one mouse in the WT/control group and one mouse from the *ob*/MCD group were outliers and were excluded from the analysis. All analyses were carried out using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

RESULTS

NASH Phenotype in MCD and *ob/ob* Models

To determine the effects of the MCD model of NASH and the *ob/ob* model of NASH and diabetes on liver and kidney pathology, hematoxylin-eosin-stained tissue sections were scored by a trained veterinary pathologist. The MCD diet

induced characteristic histopathological steatosis and inflammation features of NASH in WT mice (Fig. 1A and C). The *ob/ob* mice on the control diet had more severe steatosis but only minimal inflammation, whereas the *ob/ob* mice on the MCD diet had the most severe NASH phenotype (Fig. 1A and C). No liver fibrosis was observed in mice from any group. In contrast to liver findings, no groups had evidence of kidney injury or pathology (Fig. 1B), which is consistent with previous reports (22). To determine the presence of the diabetic phenotype, plasma glucose and insulin levels were measured in each group of mice. The MCD diet caused a reduction in the plasma glucose levels in both genotypes (Fig. 2A). The *ob/ob* mice on the control diet had dramatically higher plasma insulin levels and there was an MCD diet effect on plasma insulin levels, although the effect was much greater in the *ob/ob* genotype than in the WT mice (Fig. 2B). Furthermore, genotype caused a much lower glucose-to-insulin ratio in the *ob/ob* mice, indicating a higher degree of insulin resistance compared with the WT mice (Fig. 2C).

Metformin Exposure Is Increased in Disease Groups

To determine the effects of NASH on metformin disposition, an oral pharmacokinetic study was performed. The

plasma time course for metformin throughout the 12-h study is shown in Fig. 3A. Both diet and genotype had significant effects on the plasma AUC for metformin, although the MCD diet caused dramatically higher plasma AUC in the *ob/ob* group than the MCD diet caused in the WT group (Fig. 3B). There was a diet effect on both the plasma half-life and mean residence time, and there was also an effect of genotype on mean residence time (Fig. 3C). Oral volume of distribution was increased only in the *ob/control* group, whereas the oral clearance of metformin was decreased by the MCD diet in both WT and *ob/ob* groups (Fig. 3D). The MCD diet caused an increase in the concentration of metformin in liver, kidney, and muscle (Fig. 4). Genotype also caused an increase in metformin concentrations in the liver (Fig. 4). The percent of the metformin dose that was excreted in the feces was <10% for all treatment groups, indicating similar absorption in the intestine (Table 1). The WT/control, WT/MCD, and *ob/control* groups all excreted ≥60% of the metformin dose in the urine, whereas the *ob/MCD* group excreted only 28% of the dose during the course of the study (Table 1).

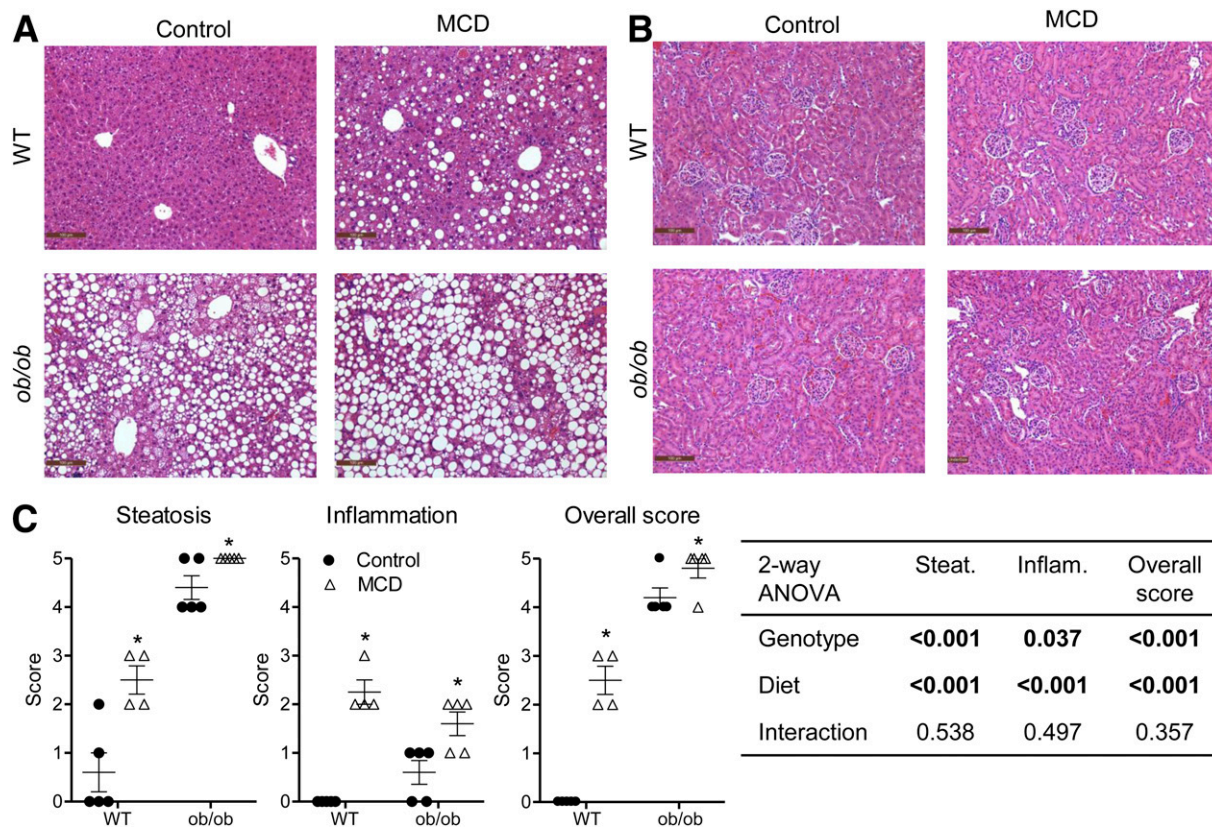


Figure 1—Liver and kidney histology in WT and *ob/ob* mice. Hematoxylin-eosin-stained liver (A) and kidney (B) sections from WT or *ob/ob* mice fed either a control or MCD diet. Original magnification ×200. C: Pathology scores were as follows: 0, no significant lesions (0%); 1, minimal (<10%); 2, mild (10–25%); 3, moderate (25–40%); 4, marked (40–50%); and 5, severe (>50%). Data represent mean ± SEM. Boldface values in the table indicate *P* < 0.05. Control, *n* = 5; MCD, *n* = 4; *ob/control*, *n* = 5; *ob/MCD*, *n* = 5. **P* value < 0.05 according to a two-way ANOVA Bonferroni posttest that was performed comparing control to MCD or *ob/control* to *ob/MCD*. Closed circles, control diet; open triangles, MCD diet.

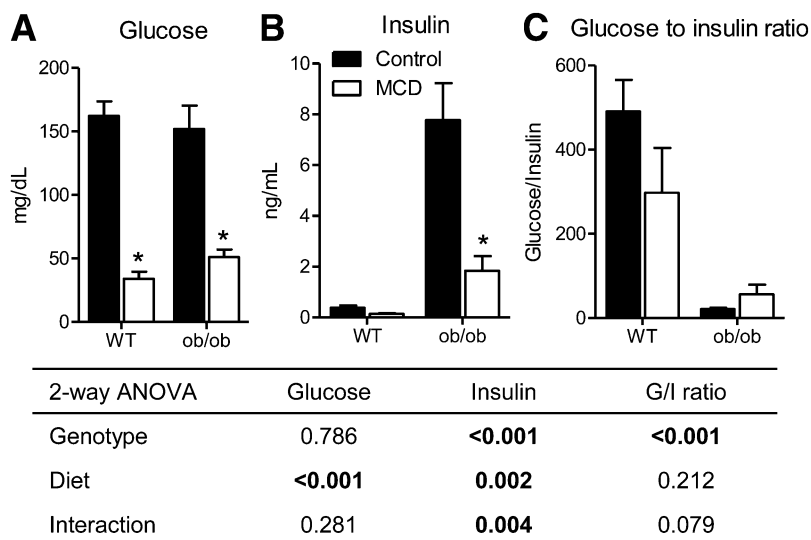


Figure 2—Effect of MCD diet and *ob/ob* strain on glucose and insulin. Plasma samples from WT or *ob/ob* mice fed either a control diet or MCD diet analyzed for glucose (A) and insulin (B) concentrations. C: The glucose-to-insulin ratio was calculated by dividing the glucose concentration by the insulin concentration for each animal. Data represent mean \pm SEM. Boldface values in the table indicate $P < 0.05$. Control, $n = 5$; MCD, $n = 4$; *ob/control*, $n = 5$; *ob/MCD*, $n = 5$. * P value < 0.05 according to a two-way ANOVA Bonferroni multiple comparison posttest. Black bars, control diet; white bars, MCD diet.

Hepatic and Renal Transporters Are Altered in Mice With Disease

Metformin is known to be a substrate for mouse Oct1, Oct2, and Mate1. Expression of hepatic Oct1 mRNA was increased in mice fed the MCD diet, whereas Mate1 mRNA was increased in the *ob/ob* mice fed either diet (Fig. 5). In the kidney, Oct1 mRNA was decreased in the *ob/ob* mice fed the MCD diet. Kidney Oct2 and Mate1 mRNA were both decreased by *ob/ob* genotype and by the MCD diet, where the lowest expression of both transporters was observed in the *ob/ob* mice fed the MCD diet (Fig. 6).

DISCUSSION

It is now accepted that NASH-associated changes in liver function and transporter expression can have a dramatic effect on drug metabolism and disposition and that there is a higher incidence of CKD in NAFLD patients, but there is a paucity of information regarding how NASH can effect kidney transporter expression and disposition of drugs excreted in the urine (10–12,14–18,23–31). Metformin is widely used for the management of T2DM and it has been suggested that the metformin dosage needs to be individualized in order to maximize therapeutic benefit and minimize adverse effects because of wide variability in pharmacokinetics (3,32). Metformin is almost entirely eliminated through the kidney and is currently being considered for its therapeutic effects on liver pathology associated with NASH. There are a number of adverse effects that are associated with metformin treatment, including the common reactions of diarrhea and nausea/vomiting and the rare and potentially fatal reaction of lactic acidosis (5). There is a great need to identify the risk factors for development of these adverse effects,

and here we show for the first time that rodent models of NASH and diabetes have extrahepatic effects on kidney transporter expression, resulting in increased systemic exposure to metformin.

At physiological pH, metformin is primarily cationic and is dependent on transporters for passage through epithelial barriers within the body. Approximately 80% of the renal clearance of metformin is accomplished by active secretion mediated by OCT1, OCT2, and MATE1 (5,6,20,33,34). The OCTs are responsible for metformin uptake from the blood and are expressed on the basolateral side of hepatocytes (OCT1 and Oct1) and proximal tubule cells (OCT2, Oct1, and Oct2), whereas MATE1/Mate1 is responsible for efflux from cells on the apical side of hepatocytes and proximal tubule cells. OCT3 can also transport metformin but due to low expression of OCT3 in liver and kidney, only marginal nonsignificant changes in metformin plasma AUC have been observed in male Oct3 knockout mice (35,36). MATE2-K can also contribute to secretion of metformin but it is not expressed in rodent kidney and therefore it was not measured in these experiments (37). Evidence from preclinical and clinical studies indicates that changes in expression and function of these transporters can dramatically impact the pharmacokinetics and pharmacodynamics of metformin.

Here we show that the combined NASH- and diabetes-associated changes in kidney expression of Oct1, Oct2, and Mate1 caused a 4.8-fold increase in plasma exposure for *ob/MCD* mice (Figs. 3 and 6). These data are in agreement with previously published data from preclinical models showing that a combination of changes in transporters is often required to elicit changes in metformin pharmacokinetics. For example, it has previously been

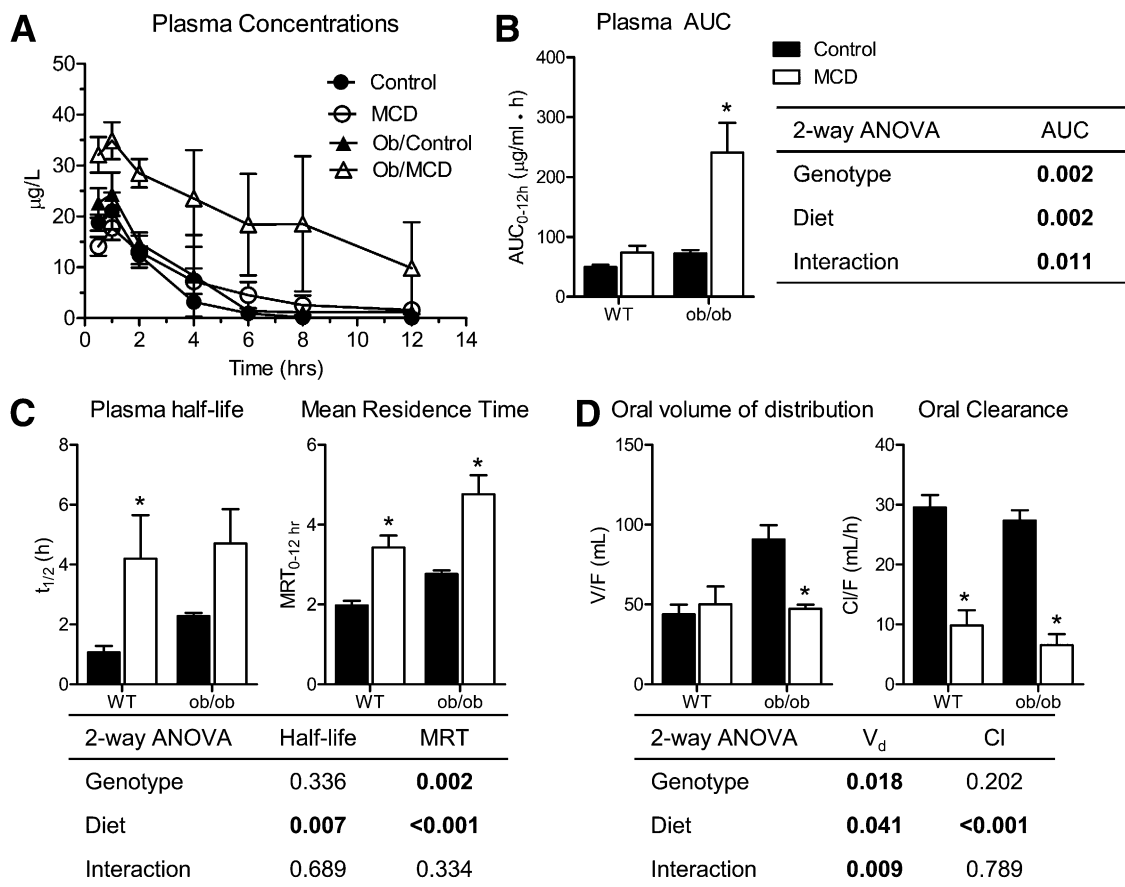


Figure 3—Altered pharmacokinetic parameters in diseased mice. Metformin was quantified in the plasma from mice dosed orally with metformin. *A*: Time course of metformin plasma concentrations. Black circles, control; white circles, MCD; black triangles, ob/control; white triangles, ob/MCD. *B*: Metformin plasma AUC. *C*: Metformin plasma half-life and mean residence time. *D*: Metformin oral volume of distribution (V/F) and oral clearance (Cl/F). Data represent mean ± SEM. Boldface values in the tables indicate *P* < 0.05. Control, *n* = 4; MCD, *n* = 4; ob/control, *n* = 5; ob/MCD, *n* = 4. **P* value < 0.05 according to a two-way ANOVA Bonferroni multiple comparison posttest. Black bars, control diet; white bars, MCD diet.

shown that Oct1 knockout alone is insufficient to cause altered pharmacokinetics but due to functional redundancy of these transporters in the mouse kidney, double knockout of Oct1 and Oct2 caused 3.8-fold higher systemic metformin exposure (6,20). It has also been shown that heterozygous knockout of *Mate1* in mice has no

effect on metformin pharmacokinetics, whereas complete knockout of *Mate1* increased metformin plasma AUC (twofold), decreased urinary excretion, decreased renal clearance, decreased renal secretory clearance, and caused severe lactic acidosis (33,34). These data are also consistent with our data showing that the MCD-associated

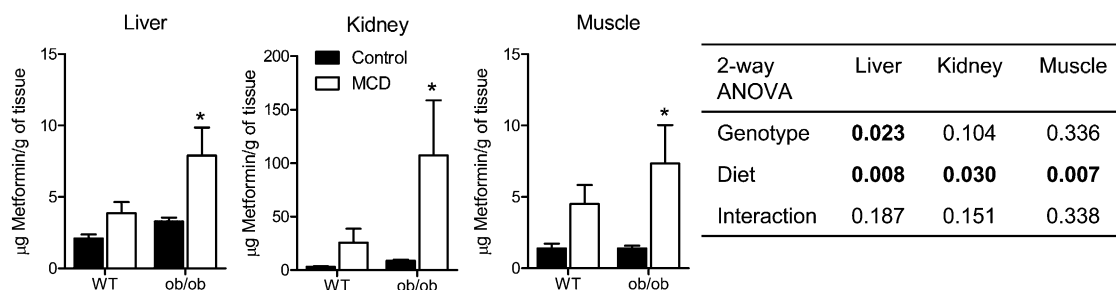


Figure 4—Metformin concentrations are increased in liver, kidney, and muscle of diseased mice. Metformin was quantified in the liver, kidney, and muscle tissues from mice dosed orally with metformin. Data represent mean ± SEM. Boldface values in the table indicate *P* < 0.05. Control, *n* = 4; MCD, *n* = 4; ob/control, *n* = 5; ob/MCD, *n* = 4. **P* value < 0.05 according to a two-way ANOVA Bonferroni multiple comparison posttest. Black bars, control diet; white bars, MCD diet.

Table 1—Percentage of metformin dose excreted in urine and feces during the course of the study

	Urine (%)	Feces (%)
WT/control	82	9
WT/MCD	60	2
ob/Control	70	8
ob/MCD	28	8

Data represent pooled urine and feces data from each treatment group.

decrease in Oct2 and Mate1 in WT mice or the *ob/ob*-associated decrease in these transporters (control diet) caused only a slight increase in plasma AUC of metformin, likely due to the compensatory activity of Oct1 in the kidney (Figs. 3 and 6). Collectively, these data suggest that these transporters work together in the clearance of metformin and should be considered together when evaluating the clinical factors that can alter metformin pharmacokinetics.

In addition to changes in plasma metformin concentrations, we also observed that the MCD diet caused increased liver, kidney, and muscle metformin concentrations in both WT and *ob/ob* strains. These data are consistent with the MCD diet-induced changes in metformin pharmacokinetics we observed (i.e., increased plasma exposure, increased plasma half-life and mean residence time, and decreased oral clearance). It is difficult to

determine the precise cause of these changes in tissue concentrations from the data presented here but they do suggest that increased systemic exposure to metformin leads to higher tissue concentrations. These higher tissue concentrations could have implications for metformin's glucose-lowering effect and toxicities, although this needs to be explored further in this model.

Numerous clinical pharmacogenetics studies have shown that single nucleotide polymorphisms (SNPs) in OCT1, OCT2, and MATE1 can work alone or in combination to alter the pharmacokinetics and pharmacodynamics of metformin. SNPs in hepatic OCT1 have been reported to increase plasma AUC, increase plasma maximal concentrations, reduce trough steady-state metformin plasma concentrations, and reduce the pharmacodynamic effect of metformin (6,32,38). Variants in OCT2 have also been shown to increase metformin plasma AUC, increase peak concentrations, and contribute to decreased oral clearance (39). For MATE1, it has been reported that individuals who are heterozygous for a series of reduced function alleles have no change in the pharmacokinetics of metformin (34). In contrast, another study found that one SNP (*rs2289669*) in MATE1 was associated with increased efficacy of metformin therapy, suggesting increased metformin exposure, although the pharmacokinetics of metformin was not evaluated in this study (40). A gene-by-gene interaction effect has been reported for MATE1 and OCT2 where the combination of the two SNPs caused a decrease in total renal clearance and renal secretory clearance (41). These data, in conjunction with the data presented here and our recent data highlighting the ability for multiple factors to interact and cause synergistic increases in drug exposure, suggest that the effect of NASH on metformin pharmacokinetics needs to be considered both alone and in combination with other factors that can increase metformin exposure (26). This assertion is supported by a recent population pharmacokinetics study that suggested that metformin may be safely administered to patients with renal impairment if the variables affecting metformin pharmacokinetics are known and appropriately accounted for (42). On the basis of the data presented here, we suggest that NASH may need to be included as one of these factors due to its effect on kidney function and potentially transporter function.

There have been a number of preclinical and clinical studies showing that metformin may be an effective treatment for the liver pathologies associated with NAFLD and NASH. However, these studies also suggest that NASH causes a nonsignificant increase in the risk of lactic acidosis that, when combined with other risk factors, may synergistically increase that risk. In clinical trials, plasma lactic acid is commonly used to monitor for the development of lactic acidosis. A number of studies have reported no adverse effects but, importantly, lactic acid was not reported (43–47). A retrospective study found that metformin improves the survival of NASH

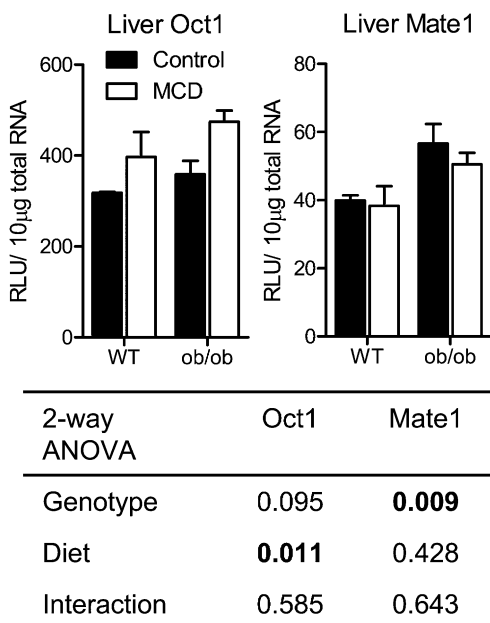


Figure 5—Hepatic transporter mRNA expression. Oct1 and Mate1 mRNA expression in the livers of WT or *ob/ob* mice fed either a control diet or MCD diet. Data represent mean \pm SEM. Boldface values in the table indicate $P < 0.05$. Control, $n = 4$; MCD, $n = 4$; ob/control, $n = 5$; ob/MCD, $n = 4$. Black bars, control diet; white bars, MCD diet. RLU = relative light units.

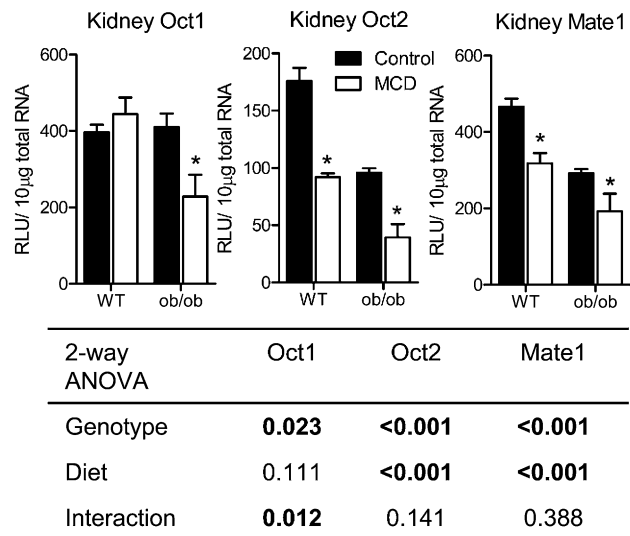


Figure 6—Kidney transporter mRNA expression is altered in diseased mice. Oct1, Oct2, and Mate1 mRNA expression in the kidneys of WT or *ob/ob* mice fed either a control diet or MCD diet. Data represent mean \pm SEM. Boldface values in the table indicate $P < 0.05$. Control, $n = 4$; MCD, $n = 4$; *ob/control*, $n = 5$; *ob/MCD*, $n = 4$. * P value < 0.05 according to a two-way ANOVA Bonferroni multiple comparison posttest. Black bars, control diet; white bars, MCD diet. RLU = relative light units.

patients with diabetes but also noted that the most common reason for the discontinuation of metformin was the concern for development of lactic acidosis (48). In studies where lactic acid was measured and reported, there appears to be an increase in lactic acid concentrations in NAFLD patients. For example, in one study there was a mild increase in lactate levels noted during treatment that generally stayed within normal limits, but 1 patient (out of 15) had to withdraw due to lactate levels that increased beyond the upper limit (49). Another study found that lactic acid increased by 30% in patients treated with metformin and exceeded the upper limit in 1 patient (out of 14) (50). A third study found that metformin treatment increased lactic acid levels in 6 patients, with 3 exceeding the upper limit (out of 36), although the increase was not statistically significant at the end of the treatment (51). One study did not measure lactic acid but did report that the combination of metformin plus pentoxifylline was poorly tolerated, causing diarrhea and nausea in 40 and 10% of patients, respectively. Two patients discontinued treatment in the first months of treatment due to toxicities and it is unclear if these adverse reactions are due to metformin, pentoxifylline, or both (52). It is important to point out that most of these studies evaluating the efficacy of metformin were not designed to, nor did they have the statistical power to, capture the overlap of one or more risk variables for altered metformin pharmacokinetics and adverse effects. To date, there have been no pharmacokinetic studies to determine if NAFLD alters the disposition of metformin and this will be an important area of future research (53). Although our

data are from a preclinical rodent model of NASH and diabetes, they accentuate the need for properly powered and designed studies to determine how NAFLD may work alone or in combination with other factors in individual variability to alter metformin pharmacokinetics and the risk of adverse effects.

In the current study, we show that the combined effects of MCD diet- and *ob/ob*-induced NASH phenotypes alter the expression of multiple kidney transporters and increase systemic exposure of metformin. The data presented suggest that as the clinical trials for metformin in NAFLD patients proceed, a careful consideration needs to be made to account for the numerous factors that can influence metformin pharmacokinetics in order to minimize the risk of adverse effects. In the absence of confirmed changes in human renal OCT2 and MATE1 transporters, the fact that impaired kidney function is known to occur in 25% of NASH patients highlights the need to account for genetic- and disease-associated changes in renal function (17). This work lays the groundwork for future studies into the safety of metformin in NAFLD patients.

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