Molecular Pharmacology Fast Forward. Published on April 28, 2015 as DOI: 10.1124/mol.114.096776
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Title Page:

Targeted Disruption of Organic Cation Transporter 3 (Oct3) Attenuates the Pharmacologic Response to Metformin

Eugene C. Chen, Xiaomin Liang, Sook Wah Yee, Ethan G. Geier, Sophie L Stocker, Ligong Chen, Kathleen M Giacomini

Department of Bioengineering and Therapeutic Sciences (E.C.C., X.L., S.W.Y., K.M.G.), University of California, San Francisco.

Current address: Apricity Therapeutics, Inc. (E.G.G.)

Current address: GlaxoSmithKline (S.L.S.), Victoria, Australia.

Department of Pharmacology and Pharmaceutical Sciences (L.C.), School of Medicine, Tsinghua University, Beijing 10084, China.

Institute for Human Genetics (K.M.G.), University of California, San Francisco.

Molecular Pharmacology Fast Forward. Published on April 28, 2015 as DOI: 10.1124/mol.114.096776
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Running Title Page:

Ablation of Oct3 Attenuates Metformin Response

Corresponding Author:

Kathleen M Giacomini

E-mail: Kathy.giacomini@ucsf.edu

University of California San Francisco

1550 4th Street

Box 2911

San Francisco, CA 94158

Tel: (415) 476-1936

Fax: (415) 514-4361

Number of text pages: 23

Number of tables: 1

Number of figures: 5

Number of references: 34

Number of words in the abstract: 243

Number of words in the introduction: 686

Number of words in the discussion: 1333

Molecular Pharmacology Fast Forward. Published on April 28, 2015 as DOI: 10.1124/mol.114.096776
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Abbreviations:

OCT, organic cation transporter; mOCT3, murine organic cation transporter 3; SLC, solute carrier; MATE, multidrug and toxin extrusion; RT-PCR, reverse transcription-polymerase chain reaction; V, apparent volume of distribution; CL, clearance; AUC, area under the concentration-time curve; F, bioavailability; 3'UTR, three prime untranslated region; eQTL, expression quantitative trait loci; HCT-116, human colorectal carcinoma cell line; HepG2, human liver carcinoma cell line.

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Abstract

Metformin, the most widely prescribed anti-diabetic drug, requires transporters to enter tissues involved in its pharmacologic action including liver, kidney and peripheral tissues. Organic cation transporter 3 (OCT3, *SLC22A3*), expressed ubiquitously, transports metformin, but its *in vivo* role in metformin response is not known. Using *Oct3* knockout mice, the role of the transporter in metformin pharmacokinetics and pharmacodynamics was determined. After an intravenous dose of metformin, a 2-fold decrease in the apparent volume of distribution and clearance was observed in knockout compared to wildtype mice ($p < 0.001$), indicating an important role of OCT3 in tissue distribution and elimination of the drug. Following oral doses, a significantly lower bioavailability was observed in knockout compared to wildtype mice (0.27 versus 0.58, $p < 0.001$). Importantly, metformin's effect on the plasma glucose concentration-time curve was reduced in knockout compared with wildtype mice (12% versus 30% reduction, respectively, $p<0.05$) along with its accumulation in skeletal muscle and adipose tissue ($p<0.05$). Further, the effect of metformin on phosphorylation of AMP activated protein kinase, AMPK, and expression of glucose transporter type 4 was absent in the adipose tissue of *Oct3*$^{-/-}$ mice. Additional analysis revealed that an *OCT3* 3'UTR variant was associated with reduced activity in luciferase assays and reduced response to metformin in 57 healthy volunteers. These findings suggest that OCT3 plays an important role in the absorption and elimination of metformin, and that the transporter is a critical determinant of metformin bioavailability, clearance, and pharmacologic action.

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Introduction

In 2012, approximately 20 million people in the United States had been diagnosed with type 2 diabetes mellitus, resulting in a substantial toll on health in the country (H.H.S, 2014). Among the array of pharmacological agents aimed at the treatment of type 2 diabetes, the biguanide, metformin is recommended for first-line oral therapy and is particularly beneficial for overweight diabetic patients (Boyle *et al.*, 2010). Although metformin has been used for decades, its mechanism of action is not completely understood. However, the drug clearly reduces the rate of ATP synthesis, and results in higher AMP/ATP ratios (Pernicova and Korbonits, 2014). One of the outcomes from the altered energy status of the cell is activation of AMP-activated protein kinase, AMPK. At least in part due to activation of AMPK, metformin decreases glucose output by the liver, which is widely considered to be the primary site of metformin action. In addition, the drug enhances glucose utilization in peripheral tissues, particularly skeletal muscle and adipose tissues (Kirpichnikov *et al.*, 2002). These changes ultimately contribute to the therapeutic effect of metformin: improved insulin sensitivity and glycemic control.

Because of its low hydrophobicity, metformin requires membrane transporters to cross biological membranes and enter and exit from cells. Previously, studies from our laboratory and others have shown that organic cation transporters 1 and 2, OCT1 (*SLC22A1*) and OCT2 (*SLC22A2*), transport metformin in the liver and kidney, respectively (Jonker *et al.*, 2001, 2003; Wang *et al.*, 2002; Chen *et al.*, 2014). For example, in *Oct1* knockout mice, the accumulation of metformin is significantly reduced

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in the liver compared to wild-type mice (Shu *et al.*, 2007). Further, healthy volunteers with reduced function variants of OCT1 exhibit reduced pharmacologic response to metformin compared to those with reference OCT1 (Shu *et al.*, 2007). On the other hand, significantly altered systemic exposure and renal clearance were observed in healthy volunteers with genetic variants of OCT2 (Song *et al.*, 2008; Chen *et al.*, 2009). OCT2 mediated drug-drug interactions have also been described (Somogyi and Muirhead, 1987; Somogyi *et al.*, 1987), though recent studies suggest that transporters in the multi-drug and toxin extrusion protein family, MATE (*SLC47A*) play more important roles in these interactions. Because of their interactions with many drugs, OCT1 and OCT2 along with MATEs have been included in drug-drug interaction guidances from regulatory authorities in Europe and the U.S.

In contrast to our knowledge of OCT1 and OCT2, much less is known about the third member of the SLC22 family, organic cation transporter 3, OCT3. The transporter exhibits overlapping substrate specificity with OCT1 and OCT2 (Koepsell *et al.*, 2003), and in particular is an excellent transporter for metformin. However unlike OCT1 and OCT2, OCT3 is expressed ubiquitously in most tissues. Originally thought to be a major component of the extraneuronal monoamine transporter system, scavenging neurotransmitters that escaped reuptake in the central nervous system, OCT3 has been shown to play an important role in the homeostasis and neuropharmacology of monoamines (Zwart *et al.*, 2001; Wultsch *et al.*, 2009; Zhu *et al.*, 2010; Horton *et al.*, 2013). Alhough OCT3 is widely expressed in many tissues and has been shown to be the most highly expressed organic cation transporter in skeletal muscle and adipose

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tissue (Bleasby *et al.*, 2006), little is known about its biological or pharmacological roles in peripheral tissues. A recent study shows that OCT3 is important in the salivary accumulation of metformin, suggesting that it plays a role in the taste-altering effects of the drug (Lee *et al.*, 2014). In this study, we hypothesized that OCT3 plays a critical role in the pharmacologic response to the drug.

Using knockout mice, the role of OCT3 in the pharmacokinetics and pharmacodynamics of metformin was determined. In particular, the pharmacokinetic profiles and tissue accumulation of metformin were compared between wildtype and *Oct3* knockout mice after intravenous and oral doses. The glucose lowering effect of metformin after an oral glucose tolerance test (OGTT) was investigated in the mice, and the role of OCT3 in metformin action in adipose tissues was further examined. Finally, the effect of OCT3 genetic polymorphisms on the pharmacologic effects of metformin was assessed in healthy volunteers.

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Materials and Methods

Animals and Materials. *Oct3* knockout mice were generated as previously described in C57BL/6J background (Vialou *et al.*, 2008) and wildtype mice were obtained from Jackson Laboratories. Animal studies described here were conducted in male *Oct3* knockout and wildtype mice (12-16 weeks old), and were reviewed and approved by UCSF IACUC. Metformin and glucose were purchased from Sigma Chemical (St. Louis, MO). Cell culture media were purchased from Life Technologies (Carlsbad, CA). All other chemicals were commercially available.

Uptake Study. The stably overexpressing human or mouse OCT3 (pcDNA5/FRT vector) cell lines were generated from HEK FlpIn-293 cells (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM H-21) supplemented with 75 ug/ml of hygromycin B penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% fetal bovine serum. For the uptake studies, cells were cultured on poly-D-lysine coated 24-well plates to 95% confluence. The cells were washed once with warm Hank's balanced salt solution (HBSS) and then incubated in the uptake buffer with various concentration of unlabeled metformin and [^{14}C] metformin (American Radiolabeled Chemicals, St. Louis, MO) in HBSS. The uptake was performed at 37°C for 3 minutes, then the cells were washed 3 times with ice-cold HBSS. The cells were lysed with lysis buffer containing 0.1 N NaOH and 0.1% SDS, and the radioactivity in the lysate was determined by liquid scintillation counting. The K_m and V_{max} were calculated by fitting the data to Michaelis-Menten equations using GraphPad Prism software (La Jolla, CA).

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Real Time RT-PCR Analysis of mRNA Level in Tissues. Total RNA from various C57BL/6J mouse tissues was isolated using RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Two µg of total RNA from each sample was reverse transcribed into cDNA using SuperScript VILO cDNA Synthesis kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Quantitative real-time PCR was carried out in 384-well reaction plates using 2X Taqman Fast Universal Master Mix (Applied Biosystems, Foster City, CA), 20X Taqman specific gene expression probes and 10 ng of the cDNA template. The reactions were carried out on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The relative expression level of each mRNA transcript was calculated by the comparative method (ΔΔCt method) normalized to the housekeeping gene glyceraldhydes-3-phospate dehydrogenase (*Gapdh*).

Pharmacokinetic Study and Tissue Distribution of Metformin. Mice were fasted for 16 hours then given a dose of 50 mg/kg metformin in saline with 0.2 µCi/g of [^{14}C]metformin via oral gavage or tail vein injection. Blood samples were collected at specific time points by tail bleeding into heparinized microhematocrit capillary tubes (Fisher Scientific, Waltham MA). The capillary tubes were centrifuged to obtain the plasma portion. Mice were sacrificed at the end of the study and tissues were collected immediately. All tissues were weighed and homogenized in Solvable (PerkinElmer, Waltham MA) overnight. The amount of metformin in tissue homogenates and plasma were measured by liquid scintillation counting. The pharmacokinetic parameters were

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obtained by fitting the raw data using a noncompartmental model with WinNonlin (Pharsight, Princeton, NJ).

Oral Glucose Tolerance Test. Age-matched *Oct3* knockout and wildtype mice were fasted overnight for 16 hours, then given saline via oral gavage followed by an oral dose of glucose (3 g/kg) 15 minutes after. Blood samples were collected at specific time points by tail bleeding, and blood glucose levels were measured with FreeStyle Lite glucometer (Abbott, Abbott Park, IL). Three days after the saline treatment, mice were fasted again for 16 hours, then given an oral dose of metformin (150 mg/kg) followed by an oral dose of glucose (3 g/kg) 15 minutes later. Blood samples and blood glucose levels were collected as described.

Western Blotting and Analysis. Age-matched *Oct3* knockout and wildtype mice (N = 3) were treated with metformin (100 mg/kg) or saline via intraperitoneal injection for 5 days. On day 5, mice were sacrificed and epididymal adipose tissues were collected. The tissues were lysed and homogenized with a tissue homogenizer in Cellytic MT buffer with cOmplete mini protease inhibitor and PhosSTOP phosphatase inhibitor following the manufacturer's protocol (Sigma Chemical, St. Louis, MO; Roche Diagnostic, Indianapolis, IN). The tissue lysates from each group were combined and stored at - 80ºC. The proteins were separated on 4-20% SDS PAGE gels and transferred to PVDF membrane (Bio-Rad, Hercules, CA). The membranes were blocked overnight at 4ºC with Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat milk. Immunoblotting was performed following standard procedure, and signals were

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detected by ECL chemiluminescence reagent (GE healthcare, Piscataway, NJ). All antibodies used were purchased from Cell Signaling Technologies following manufacturer's recommendation for dilution and incubation time (Danvers, MA). Quantification analysis was conducted with ImageJ program (*http://imagej.nih.gov/ij/*) following the software manual.

Luciferase Reporter Assay. The complete 3'UTR sequence of *OCT3* was cloned into the MCR site downstream of firefly luciferase gene in the pmirGLO vector (Promega, Madison WI). Single nucleotide polymorphisms were introduced into the reference sequence using site-directed mutagenesis with QuikChange kit (Stratagene, La Jolla CA). HCT-116 and HepG2 cells were double transfected with pmirGLO containing *OCT3* 3'UTR sequence and pGL4.73 containing Renilla luciferase gene using Lipofectamine 2000 (Life Technologies, Carlsbad CA). After 24 hours incubation period, luminescence from firefly and Renilla luciferase were measured according to manufacturer's protocol (Promega, Madison WI) as previously described (Choi *et al.*, 2011).

Association Analysis of 3'UTR Variant in *OCT3* with Metformin Response. Previously, we have described the healthy human cohort which was used to determine the effects of functional variants on metformin disposition and response (Stocker *et al.*, 2013). Using the same cohort, which has been genotyped using Illumina OmniExpress1.0 as described in our previous study (Goswami *et al.*, 2014), we evaluated the effect of a functional *OCT3* variant, rs2076828, on metformin disposition

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and response in healthy volunteers. Using the pharmacokinetic and pharmacodynamic parameters that have been ascertained previously, we determined the association of rs2076828 with (i) metformin plasma AUC (area under the curve), (ii) metformin renal clearance, (iii) metformin secretory clearance and (iv) metformin effects on oral glucose tolerance test as quantified by changes in area under the curve of plasma glucose between 0 and 2 hours after oral glucose administration. We used linear regression analysis to test the effect of the variant with each of the parameters above assuming an additive genetic effect.

Statistical Analysis. Unless otherwise noted, statistical analysis was performed by Student's *t* test to identify significant differences between various treatment groups. All experiments were conducted in several groups of knockout and wildtype mice with about 2 to 4 mice per strain in each treatment group. Experimental conditions such as dose or timing of sample collections were optimized in initial experiments. In experiments shown in the figures, we used aged matched knockout and wildtype mice with 4 mice per treatment group. The results of these experiments were confirmed in initial or subsequent studies in additional mice. Mean values are specified in the figure and table legends along with standard deviation (SD).

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Results

Murine OCT3, mOCT3, transports metformin and is expressed in tissues implicated in metformin action.

A previous study has shown that human OCT3 transports metformin and its missense variants affect metformin uptake *in vitro* (Chen *et al.*, 2010). To understand whether mOCT3 plays a role in metformin pharmacokinetics and pharmacodynamics *in vivo*, we first determined the kinetics of metformin uptake by mOCT3 and the tissue distribution of *Oct3* in mice. In cells stably transfected with *Oct3* (HEK-mOCT3), the initial rate of metformin uptake increased with concentration and was saturable (Figure 1A). Compared to kinetics of uptake in HEK cells overexpressing human OCT3, the kinetics of metformin uptake in HEK-mOCT3 exhibited a lower K_m and a significantly higher V_{max} (1.1 ± 0.1 mM vs. 1.5 ± 0.2 mM, and 67.0 ± 3.5 nmol/min/mg protein vs. 10.7 ± 0.6 nmol/min/mg protein, $p < 0.01$, respectively. Supplemental Figure 1, Table 2). The kinetic parameters in mOCT3 cell lines are comparable to values obtained by Lee et al.

To assess the expression pattern of *Oct3* in C57BL/6 mice, the mRNA levels of *Oct3* in tissues were determined by quantitative real-time PCR. As shown in Figure 1B, *Oct3* mRNA was detected in all tissues tested and at significantly higher levels in lung and adipose tissue compared with other tissues. Further analyses were performed in important tissues for metformin response including the liver, kidney, skeletal muscle, and adipose tissue to compare the mRNA levels of *Oct1, Oct2,* and *Oct3* (Figure 1C-F). In the skeletal muscle and the adipose tissue, *Oct3* mRNA transcript levels were the

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highest among the three organic cation transporters. However, the mRNA levels of *Oct3* were lower than the levels of *Oct1* in the liver, and both *Oct1* and *Oct2* in the kidney.

Metformin pharmacokinetics and tissue accumulation are altered in Oct3 knockout mice

The pharmacokinetics of metformin in wildtype and *Oct3* knockout mice were evaluated after an intravenous bolus dose of 50 mg/kg via tail vein injection (Figure 2A, Table 1). The plasma concentrations of metformin were significantly higher in knockout mice at five time points up to 60 minutes after dosing. The systemic exposure, area under the plasma concentration time curve (AUC), was 2.1-fold higher in knockout mice compared to wild-type mice (5340 ± 775 min·mcg·ml^{-1} in knockout mice versus 2500 ± 257 min·mcg·ml^{-1} in wildtype mice, $p < 0.001$). Consistent with the significantly greater exposure, the systemic clearance of metformin was significantly lower in *Oct3* knockout mice in comparison to their wildtype counterparts (8.96 ± 0.85 ml·min^{-1}·kg^{-1} in knockout mice versus 18.6 ± 1.80 ml·min^{-1}·kg^{-}1 in wildtype mice, $p < 0.001$). In addition, *Oct3* deletion modulated the distribution of metformin. In particular, the apparent volume of distribution of metformin was substantially reduced in *Oct3* knockout mice (550 ± 60.3 ml·kg^{-1} in knockout mice versus 1480 ± 218 ml·kg^{-1} in wild-type mice, $p < 0.001$). Consistent with its reduced volume of distribution, metformin tissue-to-plasma ratios (2 hours after intravenous dosing) were significantly lower in the liver and adipose tissue in knockout mice (Figure 2B). However, the ratio was similar in the skeletal muscle

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between wildtype and knockout mice. Unexpectedly, *Oct3* deletion resulted in a significantly higher tissue-to-plasma ratio of metformin in the kidney.

The pharmacokinetics of metformin after oral dosing was also studied in wildtype and *Oct3* knockout mice (Figure 3A, Table 1). After a bolus dose of 50 mg/kg of metformin via oral gavage, the plasma concentrations were not significantly different between wildtype and knockout mice when followed for 4 hours after dosing, although a trend for a higher Cmax was observed in the knockout mice. Oral exposure was not significantly different between wildtype and knockout mice. Interestingly, oral bioavailability calculated from the intravenous and oral area under the curve (AUC) showed a 2.2-fold decrease in the knockout mice (0.27 ± 0.04 in knockout mice versus 0.58 ± 0.09 in wildtype mice, $p < 0.001$) suggesting that Oct3 contributes to metformin absorption. Tissue-to-plasma ratios were determined 4 hours after oral administration. The ratios in knockout mice were significantly lower in the liver, kidney, adipose tissue, and skeletal muscle (Figure 3B-F).

The effect of metformin on oral glucose tolerance is reduced in Oct3 knockout mice

The glucose lowering effect of metformin after an oral glucose tolerance test was determined in wildtype and knockout mice. In wildtype mice, the effect of metformin was apparent, i.e., the drug significantly reduced blood glucose levels at various times (i.e., at 15, 30, 60, 90, and 120 minutes) after oral glucose administration (Figure 4B). In

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contrast, though slight decreases in blood glucose levels were observed in knockout mice following metformin administration, the blood glucose levels were not significantly reduced at any of the time points tested (Figure 4A). *Oct3* deletion had no effect on baseline glucose tolerance as the blood glucose AUCs in saline treated groups were comparable between wildtype and knockout mice. On average, metformin significantly decreased glucose AUC in wildtype mice by 30% (18600 ± 2080 $mg \cdot min \cdot dl^{-1}$ in metformin treated mice versus 26000 ± 1450 $mg \cdot min \cdot dl^{-1}$ in saline treated mice, $p < 0.01$). While it did not reach significance, metformin on average decreased glucose AUC in knockout mice by 12%.

To investigate the mechanisms by which *Oct3* deletion modulated the effect of metformin on oral glucose tolerance, tissue accumulation of metformin was determined 30 minutes after oral administration of metformin (150 mg/kg). *Oct3* deletion clearly resulted in lower liver to plasma ratios of metformin at later times following both intravenous (Figure 2B) and oral (Figure 3B) doses. However at 30 minutes following a high oral dose of the drug (150 mg/kg), differences in hepatic uptake between wildtype and knockout mice were not apparent (Figure 4C) possibly reflecting a lower contribution of the transporter to the hepatic uptake of metformin at these higher concentrations. On the other hand, tissue accumulation of metformin was significantly decreased in skeletal muscle and adipose tissue (Figure 4E,F). The effect was most prominent in the adipose tissue, where the absolute exposure was decreased by 3.2-fold. Decreased accumulation of metformin in the kidney was also observed at 30 minutes in knockout mice, however it did not reach statistical significance (Figure 4D).

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No significant differences were found in the intestinal accumulation of metformin between *Oct3* knockout and wildtype mice (data not shown).

We performed further experimentation to understand the impact of reduced metformin accumulation in the adipose tissue on pharmacologic action. Wildtype and knockout mice were treated with intraperitoneal doses of metformin (100 mg/kg) for 5 days. On day 5, adipose tissues were collected, and selected proteins level and phosphorylation status were analyzed with Western blot (Figure 4G). The phosphorylated AMPK/total AMPK ratios were significantly higher (1.6 times, $p < 0.05$) in wildtype mice treated with metformin compared to wildtype mice treated with saline, consistent with metformin's known effects on AMPK (supplemental figure 2A). The pAMPK/AMPK ratios were higher (1.2 times) in knockout mice treated with metformin compared to knockout mice treated with saline. However, the difference did not reach statistical significance. No difference was observed in phosphorylated total AMPK level among all groups of mice. The data shown in Supplemental Figure 2A represent the mean ± SD of image analysis of Western blots performed in protein extracted from adipose tissue of three different mice in which metformin was dosed. Though attempts to measure GLUT4 levels on the plasma membrane were not successful, possibly due to the high fat content of adipocyte, the level of total GLUT4, a glucose transporter, was significantly increased (1.3 times, $p < 0.05$) in wildtype mice treated with metformin compared to wildtype mice treated with saline (supplemental figure 2B). The total GLUT4 level was not significantly increased in the knockout mice treated with metformin. These data suggest that mOCT3 modulates the pharmacologic action of metformin on AMPK and GLUT4 in adipose

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tissue, and are consistent with the observed effects of metformin on oral glucose tolerance in wildtype and *Oct3* knockout mice.

The OCT3 3'UTR variant (C>G) in healthy human volunteers is linked to change in metformin pharmacodynamics.

Luciferase assays were used to determine the effect of human genetic variants in the 3'UTR region of *OCT3*. The entire length of the 3'UTR region of *OCT3* was cloned into a luciferase reporter vector. Single nucleotide polymorphisms (SNPs) were introduced into vectors, and vectors were then used to transfect human colorectal carcinoma cell line, HCT-116, human hepatocellular carcinoma cell line, HepG2, human prostate cancer cell line, DU145, and human lung carcinoma cell line, A549. Luciferase activity was used as a surrogate for gene expression level. Of 11 SNPs tested (data not shown), rs2076828 had an effect on luciferase activity, where the minor allele, G, exhibited significantly lower activity compared with the reference allele in HCT-116, DU145 cell lines ($p < 0.01$, Figure 5A) and A549 cell line ($p < 0.05$). A similar trend was observed in HepG2 cell line, but the activity differences between the minor and major alleles did not reach statistical significance. The effect of the SNP on *OCT3* expression level was confirmed in expression quantitative trait loci (eQTL) analysis from previously published data (Grundberg *et al.*, 2012), where the mRNA level of *OCT3* was significantly lower in adipose tissues of subjects with the minor allele ($p < 0.01$, Figure 5B).

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We then analyzed the impact of rs2076828 on metformin pharmacokinetics and pharmacodynamics using previously published data from a healthy human cohort (N = 57) (Stocker *et al.*, 2013). The variant had no significant effect on metformin pharmacokinetic parameters, even after adjusting for creatinine clearance, age and gender (data not shown). However, analysis of pharmacodynamic data showed that the variant was associated with reduced response to metformin during oral glucose tolerance test in the healthy volunteers (Figure 5C). The healthy volunteers with the minor G allele had significantly smaller changes in their glucose AUC (mean ± SD, CC, -88±40 mg/dl/h; CG, -34±51 mg/dl/h; GG, -41±72 mg/dl/h; One-way ANOVA $p < 0.001$). We also noted that volunteers with the minor allele had significantly lower glucose AUC even before metformin dosing (mean ± SD, CC, 376±62 mg/dl/h; CG, 348±55 mg/dl/h; GG, 330±41 mg/dl/h; $p < 0.05$) suggesting an effect of *OCT3* on baseline oral glucose tolerance. However, after adjusting for the difference in glucose AUC before metformin dosing, the linear regression analysis showed that the variant remained significantly associated with metformin response ($p < 0.05$).

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Discussion

The major findings of this study are that mOCT3 modulates the pharmacokinetics and pharmacologic effects of metformin. In particular, *Oct3* deletion in mice affected metformin absorption and clearance as well as its accumulation in tissues, notably skeletal muscle and adipose tissue. The decreased accumulation of metformin in these tissues may have accounted for the strikingly reduced effects of metformin on blood glucose levels after an oral glucose challenge in *Oct3* knockout mice compared with wildtype mice. Consistent with our studies in mice, our analysis also showed that a reduced function genetic polymorphism in the 3'UTR of *OCT3* was associated with decreased response to metformin in healthy volunteers. To the best of our knowledge, this is the first study that demonstrates a critical role of OCT3 in the bioavailability, clearance, and pharmacodynamics of metformin.

Unlike many hydrophilic compounds that distribute primarily into body water spaces (Rowland and Tozer, 1995), the volume of distribution of metformin reported in the literature is large and variable (63-276L), consistent with extensive tissue accumulation (Graham *et al.*, 2011). Because of its hydrophilicity, metformin requires transporters to cross plasma membranes and enter body tissues. Transporters implicated in metformin tissue distribution include OCT1, which is expressed primarily in the liver and OCT2, expressed largely in the kidney (Jonker *et al.*, 2001, 2003). In addition, knockout mouse studies indicate that MATE1 plays an important role in renal and hepatic distribution of the drug (Tsuda *et al.*, 2009). A recent study showing that OCT3 is involved in the

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salivary gland accumulation of metformin suggests that the transporter plays a role in the taste-altering effects of the drug (Lee *et al.*, 2014).

In this study, we reaffirm the hypothesis that OCT3 is an important metformin transporter *in vivo*. The wide tissue distribution of OCT3 in mice (see Figure 1B) including adipose tissue and skeletal muscle suggests a mechanism for tissue uptake of metformin. Indeed, after intravenous administration of the drug, *Oct3* knockout mice exhibited a 2 -fold reduction in V_d (Table 1), suggesting a lower overall tissue accumulation of metformin. The reduced tissue accumulation of metformin in the knockout mice was confirmed in the liver and adipose tissue collected 2 hours after intravenous administration (Figure 2B,D). In addition to its effect on metformin tissue distribution, *Oct3* deletion also resulted in a notable reduction in metformin clearance (Table 1). Interestingly, there was a significant increase in the renal accumulation of metformin in the *Oct3* knockout mice after the intravenous metformin dose (Figure 2C). These data are similar to data obtained for metformin in *Mate1* knockout mice, that is, there is increased renal accumulation of metformin in the kidney of the *Mate1* knockout mice (Tsuda *et al.*, 2009). Our parallel results suggest that similar to mouse MATE1, mOCT3 may act as an efflux transporter for the drug in the kidney.

In contrast to the striking differences in the pharmacokinetics of metformin after intravenous administration of the drug, no significant differences in AUC, CL/F and V/F between knockout and wildtype mice were observed after oral administration (Table 1). Since AUC is determined by both oral bioavailability and systemic clearance, the data

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may be explained by an effect of *Oct3* on both bioavailability and clearance. That is, *Oct3* deletion results in a reduced clearance of metformin as noted after intravenous doses (Table 1), and a reduced bioavailability of the drug. In particular, a mean oral bioavailability of 0.58 was estimated for the wildtype mice, a value that closely matches data from a previously published study (Higgins *et al.*, 2012). In contrast, a mean oral bioavailability of 0.27 was estimated in knockout mice, suggesting that mOCT3 plays an important role in the absorption of metformin. In fact, OCT3 has been localized to the brush border membrane of the small intestine (Müller *et al.*, 2005), consistent with a role in metformin absorption. The dual effects of *Oct3* deletion on both bioavailability and clearance resulted in no apparent effect of gene deletion on AUC of metformin after oral administration of the drug.

Though no differences in the systemic plasma levels of metformin were observed after the oral dose, tissue levels of metformin were significantly lower in liver, kidney, adipose tissue and muscle of the knockout mice in comparison to the wildtype mice (Figure 3). Interestingly, in contrast to the higher kidney levels of metformin observed after the intravenous dose, renal accumulation of metformin was significantly lower in the *Oct3* knockout mice in comparison to the wildtype mice after the oral dose. These differences may reflect kinetic differences since tissue levels, which were measured at 4 hours after the oral dose, were substantially lower than those measured at 2 hours after the intravenous dose. Thus at low metformin levels, OCT3 may contribute primarily to renal uptake whereas at high metformin levels, OCT3 may contribute to metformin efflux in the kidney.

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Recently, Lee *et al.* published a study examining OCT3 tissue distribution in mice and the effect of *Oct3* deletion in metformin pharmacokinetics and tissue distribution. Similar to our study, Lee *et al.* observed no statistically significant differences in metformin AUC, CL/F, and V/F between wildtype and knockout mice after oral administration. While Lee *et al*. reported significant higher C_{max} in knockout mice, the difference between knockout and wildtype mice did not reach statistical significance in our study potentially because different doses were used. Lee *et al.* administered 15 mg/kg whereas we administered 50 mg/kg of metformin. Because of major differences in the experimental design, it is difficult to directly compare tissue distribution patterns of metformin between our study and that of Lee *et al.* In general, the skeletal muscle levels of metformin were significantly reduced in the Oct3 knockout mice in both studies. However, liver and kidney levels were reduced in our study but not in the study of Lee et al. possibly reflecting differences in the dose of metformin and the timing of tissue collection.

Several studies have used knockout mice to determine the role of membrane transporters in metformin disposition. However, few have used knockout mice to evaluate the impact of transporters on the therapeutic action of metformin. Shu *et al* reported lowered fasting plasma glucose levels in wildtype but not *Oct1* knockout mice after metformin treatment and suggested that reduced hepatic accumulation of metformin due to *Oct1* deletion resulted in reduced effects on gluconeogenesis in the liver (Shu *et al.*, 2007). In our study, metformin treatment significantly reduced blood

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glucose AUC after an oral glucose tolerance test in wildtype but not *Oct3* knockout mice (Figures 4A and B), suggesting an important role for *Oct3* in the glucose lowering effect of metformin. These differences could not be explained by differences in systemic plasma levels, since those were virtually identical in the knockout and wildtype mice (Figure 3A). However, the differences could be explained by substantial differences in metformin levels in peripheral tissues that are sites of glucose uptake and utilization (adipose tissue and skeletal muscle) between wildtype and knockout mice (Figure 4E,F). Correspondingly, phosphorylated AMPK and expression levels of the insulin-sensitive glucose transporter, GLUT4, increased in response to metformin in wildtype mice, but with barely detectable increases in knockout mice (Figure 4G). Taken together, our data suggest that OCT3 plays a major role in the uptake of metformin into adipose tissue thereby modulating the therapeutic effect of metformin.

Based on our finding that mOCT3 played a role in metformin disposition and response, we hypothesized that functional genetic polymorphisms of *SLC22A3 (OCT3)* affect metformin disposition and response. We selected an *OCT3* 3'UTR variant for the association study because it exhibited reduced function in *in vitro* luciferase assays and was associated with expression level differences in *OCT3* in eQTL analysis of adipose tissue (Grundberg *et al.*, 2012). We observed that the minor allele G was associated with reduced response to metformin in healthy volunteers (Figure 5C), consistent with lower *OCT3* mRNA levels observed previously in adipose tissue (Grundberg *et al.*, 2012) and lower luciferase activity in cell lines (Figure 5A). The fact that we did not observe a significant effect of this 3'UTR variant on metformin disposition after oral

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doses is also consistent with our *in vivo* studies in the mice suggesting no effect of *Oct3* deletion on the overall drug levels of metformin after oral administration. Collectively, the data in healthy volunteers support the findings in mice and indicate that OCT3 is an important determinant of metformin response. Similar to OCT1, OCT2 and MATEs (Jonker and Schinkel, 2004; Koepsell, 2004; Becker *et al.*, 2009), our results suggest that OCT3 is an important site for drug-drug interactions and that future studies examining OCT3-mediated drug interactions with metformin should be performed.

In conclusion, this study demonstrates that murine OCT3 plays a critical role in the clearance and bioavailability of metformin as well as its accumulation in various tissues, notably skeletal muscle and adipose tissue. The transporter has route-dependent effects on the systemic plasma levels of metformin because of dual effects on the bioavailability and clearance of the drug. Importantly, our study shows that OCT3 plays a major role in the pharmacologic response to metformin in both mice and humans.

Molecular Pharmacology Fast Forward. Published on April 28, 2015 as DOI: 10.1124/mol.114.096776
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Acknowledgement

The genotying using the Illumina HumanOmniExpress array is supported by the NIH Pharmacogenomics Research Network (PGRN)-RIKEN Global Alliance. In particular, we would like to acknowledge Dr. Michiaki Kubo and his group at RIKEN Center for Integrative Medical Sciences (Yokohama, Japan).

Molecular Pharmacology Fast Forward. Published on April 28, 2015 as DOI: 10.1124/mol.114.096776
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Authorship Contributions

Participated in research design: E.C. Chen, L. Chen, and Giacomini.

Conducted experiments: E.C. Chen, Liang, Geier, and Stocker.

Performed data analysis: E.C. Chen, Liang, Yee, and Giacomini.

Wrote or contributed to the writing of the manuscript: E.C. Chen, Liang, Yee, Geier, and Giacomini.

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Molecular Pharmacology Fast Forward. Published on April 28, 2015 as DOI: 10.1124/mol.114.096776
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Footnotes

This work was supported by the National Institutes of Health (NIH) National Institute of General Medical Sciences [GM61390] and the Burroughs Wellcome Fund Innovation in Regulatory Science Awards (IRSA) [BWF ID #1012485]; EC Chen and Liang are supported in part by the National Institute of Health Training Grant [Grant T32 GM007175].

Molecular Pharmacology Fast Forward. Published on April 28, 2015 as DOI: 10.1124/mol.114.096776
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Figure Legends.

Figure 1. Characterization of the kinetics and tissue levels of mouse OCT3. (A) Overexpressing mouse OCT3 increases metformin uptake in HEK cells. Metformin uptake studies were conducted in HEK cells overexpressing mouse OCT3 (■) or empty vector (●). Cells were incubated with increasing concentrations of metformin for 3 minutes. The uptake kinetic parameters (see Results section) were calculated using the difference in accumulation between OCT3 overexpressing and empty vector cells. (B) *Oct3* mRNA expression pattern was assessed in C57/B6J mice. The relative mRNA levels of *Oct3* were determined by real-time PCR. The mRNA levels of *Oct1, Oct2* and *Oct3* were determined in the liver (C), kidney (D), adipose tissue (E), and skeletal muscle (F) Data represent mean ± SD, *n* = 3 mice per group.

Figure 2. *Oct3* deletion resulted in altered pharmacokinetics and tissue accumulation of metformin in mice. (A) The plasma metformin concentration-time profiles in wild-type and knockout mice after an i.v. dose (50mg/kg metformin containing 0.2mCi/Kg [^{14}C]metformin via tail vein). Significantly higher plasma concentrations were observed in knockout mice at 5, 10, 15, 30, 60 minutes after injection. (B-E) Tissue distribution of metformin in wildtype and knockout mice. Two hours after dosing, mice were sacrificed and tissues were collected. Radioactivity in tissue homogenates were determined and converted to mass amounts. Tissue accumulation was significantly reduced in liver and adipose tissue of knockout mice. Metformin accumulation was higher in kidney of

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knockout mice. Data represent mean ± SD, *n* = 4 mice per data points, **p* < 0.05 wild-type versus knockout mice.

Figure 3. *Oct3* deletion resulted in altered tissue accumulation of metformin after an oral dose. (A) The plasma metformin concentration-time profiles in wild-type and knockout mice after an oral dose (50 mg/kg metformin containing 0.2mCi/Kg [^{14}C]metformin via oral gavage). Radioactivity in the plasma is determined and converted to mass amounts. (B-E) Tissue distribution of metformin in wildtype and knockout mice. Four hours after administration, mice were sacrificed and tissues were collected. Radioactivity in tissue homogenates were determined and converted to mass amounts. Metformin accumulation was significantly reduced in liver, kidney, skeletal muscle and adipose tissue of *Oct3* knockout mice. Data represent mean ± SD, *n* = 4 mice per data point, **p* < 0.05, ***p* < *0.01* wildtype versus knockout mice.

Figure 4. *Oct3* deletion resulted in reduced pharmacologic effects of metformin. The blood glucose concentration-time profiles of knockout mice treated with metformin or saline (A) and wildtype mice treated with metformin or saline (B). Mice were given metformin (150 mg/kg) or saline orally followed by glucose (3g/kg) orally after 15 minutes. Data represent mean ± SD, n = 4 mice per data point, **p* < 0.05 metformin treated group versus saline treated group. (B-E) Tissue distribution of metformin in wildtype and knockout mice. Thirty minutes after metformin (150 mg/kg p.o.), mice

were sacrificed and tissues were collected. Radioactivity in tissue homogenates were determined and converted to mass amounts. Data represent mean ± SD, n = 4 mice per data point, *p < 0.05 wildtype versus knockout mice. (G) Representative Western Blots of adipose tissues from knockout and wildtype mice treated with i.p 100mg/kg metformin or saline for 5 days.

Figure 5. Functional characteristics of an OCT3 3'UTR variant (rs2076828) and its association with metformin pharmacologic response in healthy volunteers. The OCT3 3'UTR variant (C>G) in healthy human volunteers is associated with changes in metformin pharmacodynamics. (A) Luciferase reporter assay of rs2076828. Luciferase activity was significantly lower in HCT-116, DU145, and A549 cell lines transfected with a reporter construct containing the minor allele (G) compared with the reference allele (C). Data represent mean ± SD, n = 3 replicates per cell line, *p <0.05 and **p < 0.01. (B) E-QTL analysis of rs2076828 in healthy human cohorts revealed lower mRNA level of OCT3 in adipose tissues of subjects with the minor allele (G), p < 0.01. (C) The change in glucose exposure following an oral glucose tolerance test (OGTT) (area under the curve, AUC) after metformin treatment in healthy volunteers. Change in glucose AUC was calculated as glucose AUC after metformin dosing minus glucose AUC before metformin dosing. Healthy volunteers with the minor allele had lower response to metformin (p < 0.05). The dot plot displays the mean ± SD.

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Tables.

Table 1. Pharmacokinetic parameters of metformin in *Oct3* knockout and wildtype mice.

IV Parameter	**Wild-type**	***Oct3* Knockout**
C_0 (mcg/ml)	70.9 ± 11.7	148 ± 18.7 **
AUC (min*mcg/ml)	2500 ± 257	5340 ± 775 *
V (ml/kg)	1480 ± 218	550 ± 60.3 *
CL(ml/min/kg)	18.6 ± 1.80	8.96 ± 0.85 ***
Oral Parameter	**Wild-type**	***Oct3* Knockout**
Cmax (mcg/ml)	9.70 ± 0.77	11.2 ± 0.39
AUC (min*mcg/ml)	1460 ± 184	1480 ± 44.5
V/F (ml/kg)	4240 ± 698	3670 ± 293
CL/F (ml/min/kg)	27.4 ± 2.25	28.0 ± 0.45
F (AUC Oral/AUC IV)	0.58 ± 0.09	0.27 ± 0.04

Oral bioavailability was obtained by dividing AUC_{oral} by AUC_{IV}. Each value represents the mean ± S.D. for four mice.

* $P < 0.05$, significantly different from wildtype mice.

** $P < 0.01$, significantly different from wildtype mice.

*** $P < 0.001$, significantly different from wildtype mice.

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Table 2. Kinetic parameter of metformin uptake in HEK293 cells expressing mouse and human OCT3.

	hOCT3	mOCT3
K_m (mM)	1.5 ± 0.2	1.1 ± 0.1
V_{max} (nmol/mg protein/ min)	10.7 ± 0.6	66.9 ± 3.5
K_m/V_{max} (min·mg protein/ml)	143.6 ± 20.9	16.9 ± 2.3

K_m and V_{max} Values were determined from non-linear regression fit of data from Figure 1 and Supplemental Figure 1. Data represent mean ± SD, n = 3 replicates per data point.

Figure 1

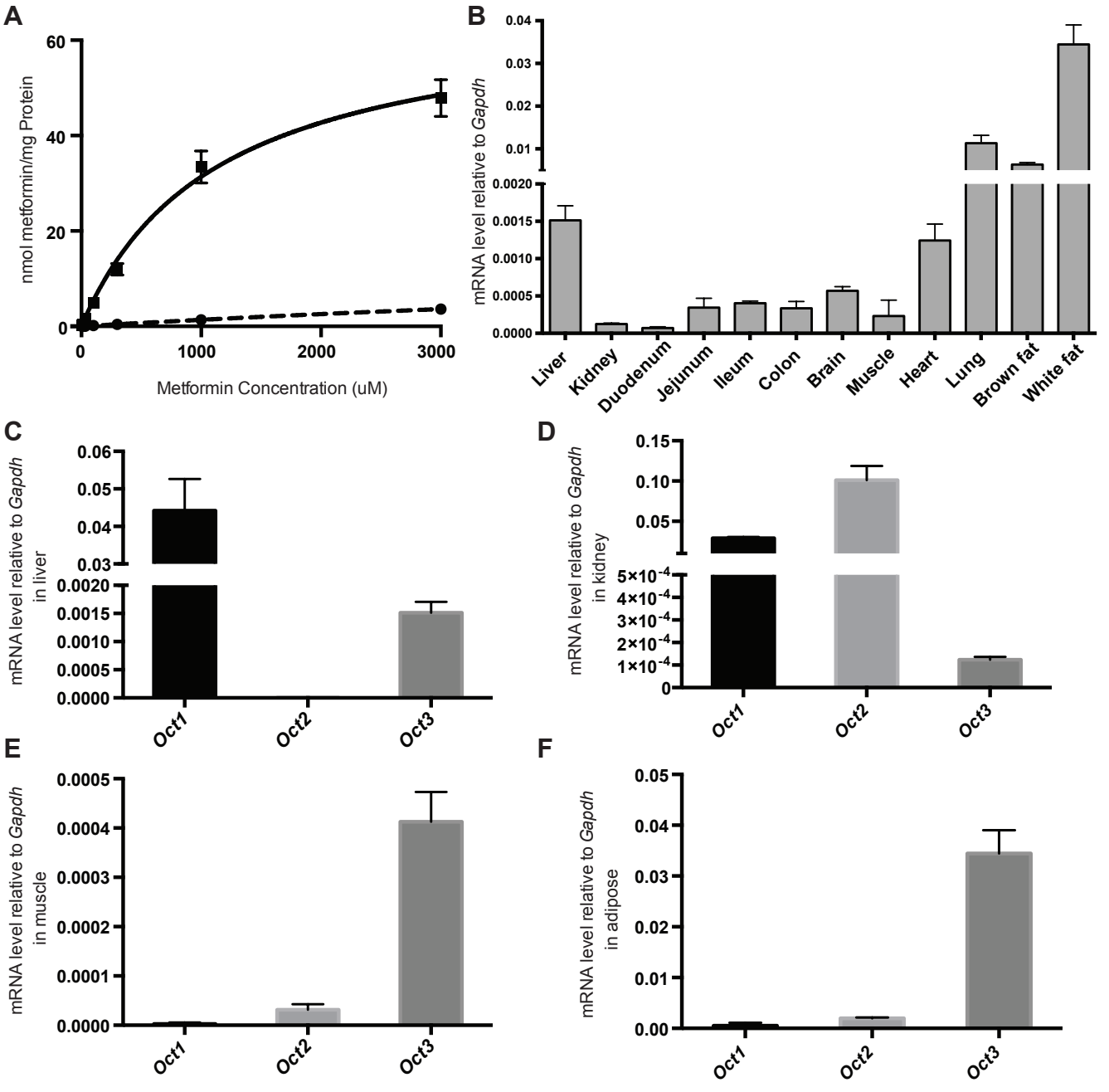

A
nmol metformin/mg Protein
60
40
20
0
0
1000
2000
3000
Metformin Concentration (uM)
B
mRNA level relative to Gapdh
0.04
0.03
0.02
0.01
0.0020
0.0015
0.0010
0.0005
0.0000
Liver
Kidney
Duodenum
Jejunum
Ileum
Colon
Brain
Muscle
Heart
Lung
Brown fat
White fat
C
mRNA level relative to Gapdh in liver
0.06
0.05
0.04
0.03
0.0020
0.0015
0.0010
0.0005
0.0000
Oct1
Oct2
Oct3
D
mRNA level relative to Gapdh in kidney
0.15
0.10
0.05
5×10-4
4×10-4
3×10-4
2×10-4
1×10-4
0
Oct1
Oct2
Oct3
E
mRNA level relative to Gapdh in muscle
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
Oct1
Oct2
Oct3
F
mRNA level relative to Gapdh in adipose
0.05
0.04
0.03
0.02
0.01
0.00
Oct1
Oct2
Oct3

Figure 2

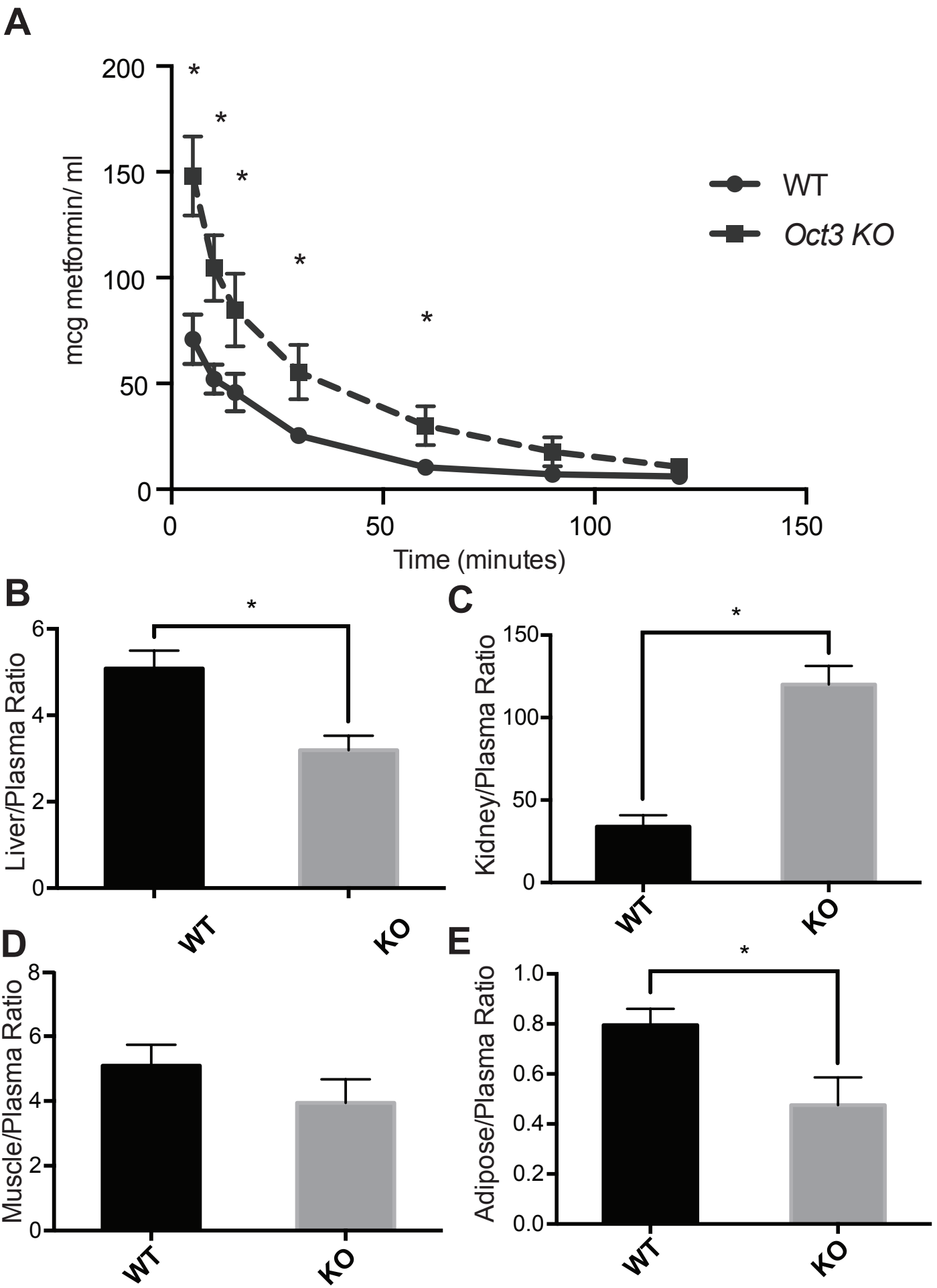

A
200
150
100
50
0
mcg metformin/ ml
*
*
*
*
*
0
50
100
150
Time (minutes)
WT
Oct3 KO
B
6
4
2
0
Liver/Plasma Ratio
*
WT
KO
C
150
100
50
0
Kidney/Plasma Ratio
*
WT
KO
D
8
6
4
2
0
Muscle/Plasma Ratio
WT
KO
E
1.0
0.8
0.6
0.4
0.2
0.0
Adipose/Plasma Ratio
*
WT
KO

Figure 3

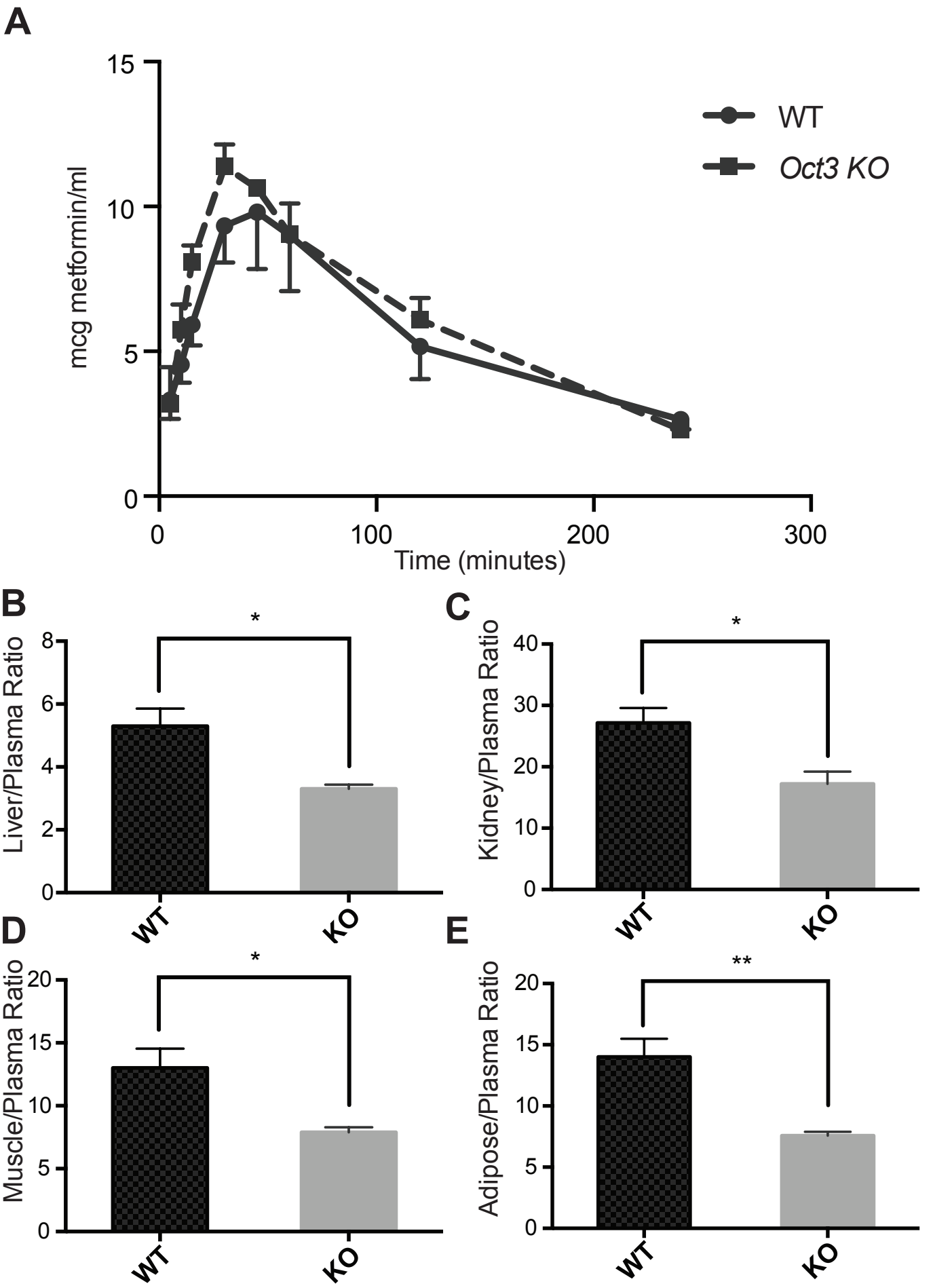

A
15
10
5
0
mcg metformin/ml
WT
Oct3 KO
0
100
200
300
Time (minutes)
B
*
8
6
4
2
0
Liver/Plasma Ratio
WT
KO
C
*
40
30
20
10
0
Kidney/Plasma Ratio
WT
KO
D
*
20
15
10
5
0
Muscle/Plasma Ratio
WT
KO
E
**
20
15
10
5
0
Adipose/Plasma Ratio
WT
KO

Figure 4

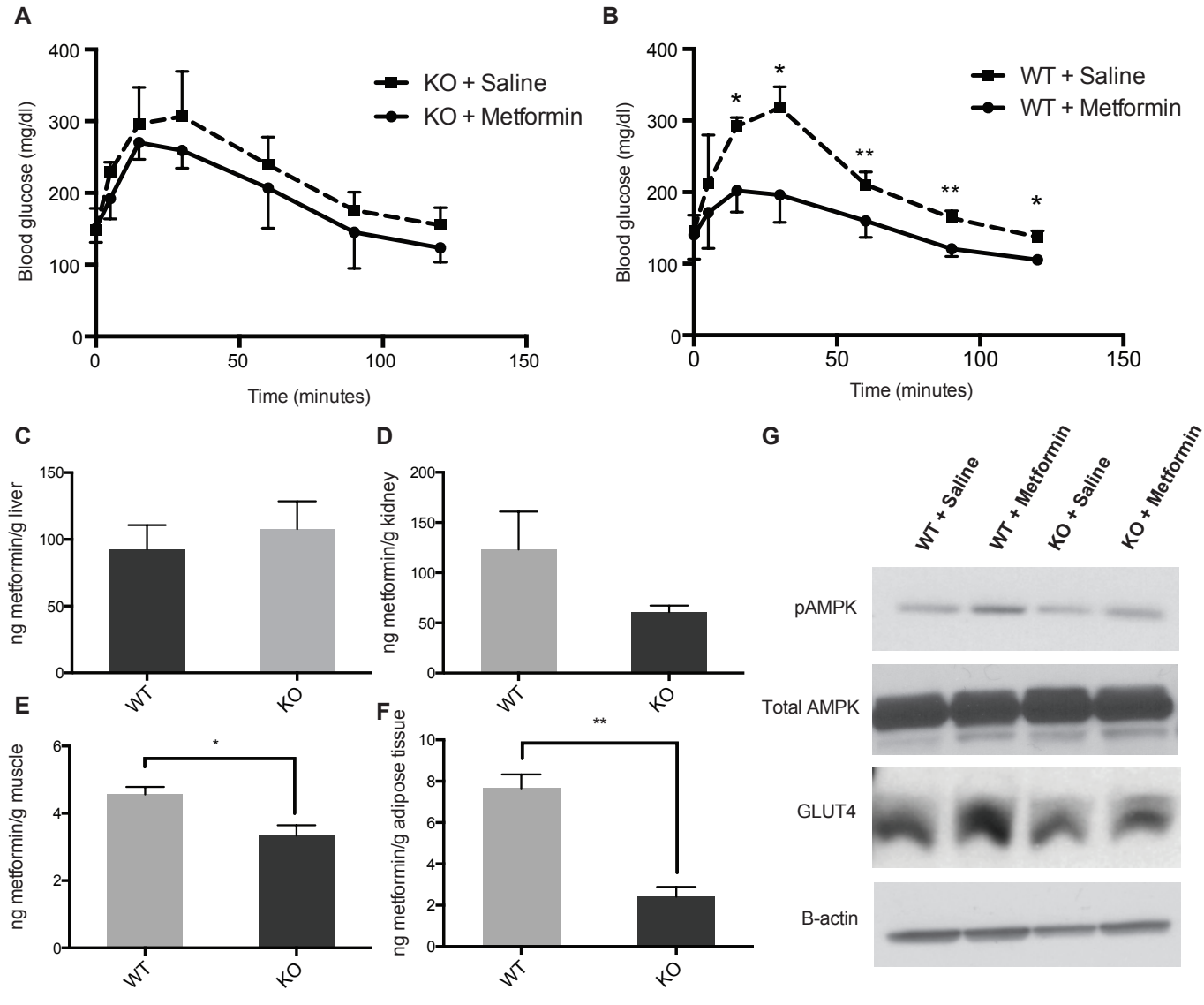
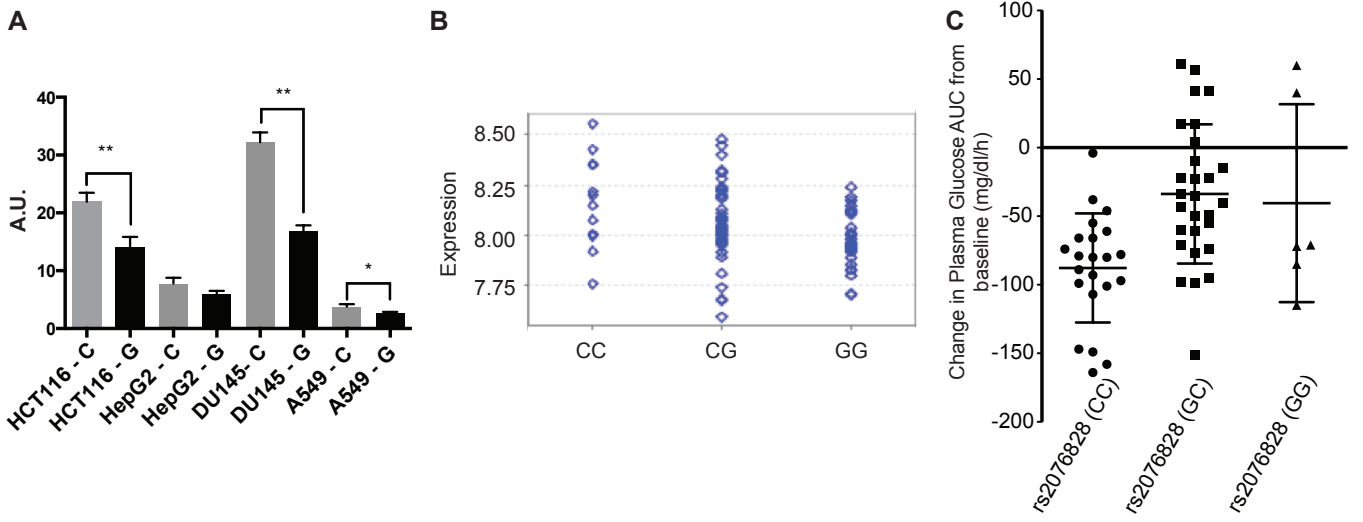

A
Blood glucose (mg/dl)
400
300
200
100
0
0
50
100
150
Time (minutes)
KO + Saline
KO + Metformin
B
Blood glucose (mg/dl)
Time (minutes)
WT + Saline
WT + Metformin
*
**
C
ng metformin/g liver
150
100
50
0
WT
KO
D
ng metformin/g kidney
200
150
100
50
E
ng metformin/g muscle
6
4
2
F
ng metformin/g adipose tissue
10
8
G
WT + Saline
WT + Metformin
KO + Saline
KO + Metformin
pAMPK
Total AMPK
GLUT4
B-actin

Figure 5


A
A.U.
40
30
20
10
0
**
**
*
HCT116 - C
HCT116 - G
HepG2 - C
HepG2 - G
DU145- C
DU145 - G
A549 - C
A549 - G
B
Expression
8.50
8.25
8.00
7.75
CC
CG
GG
C
Change in Plasma Glucose AUC from baseline (mg/dl/h)
100
50
0
-50
-100
-150
-200
rs2076828 (CC)
rs2076828 (GC)
rs2076828 (GG)