Published in final edited form as:

Mol Pharm. 2013 July 1; 10(7): 2578–2587. doi:10.1021/mp400013t.

Ischemia/Reperfusion-Inducible Protein Modulates the Function of Organic Cation Transporter 1 and Multidrug and Toxin Extrusion1

QING LI^{1,2}, HYEKYUNG YANG¹, XIUJUAN PENG¹, DONG GUO², ZHONGQI DONG¹, JAMES E. POLLI¹, and YAN SHU^{1,*}

¹Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland at Baltimore, Maryland, USA

²Institute of Clinical Pharmacology, Central South University, Hunan 410078, China

Abstract

The recently identified ischemia/reperfusion-inducible protein (IRIP) has been reported to negatively modulate the activities of several transporters in cell culture systems. The goal of this study is to determine whether IRIP regulates the activities of OCT1 and MATE1, and hence the disposition *in vivo* of their substrate metformin, a therapeutic drug for diabetes and other obesity-related syndromes. In the uptake studies in the human embryonic kidney 293 cells overexpressing IRIP with and without OCT1 or MATE1, IRIP overexpression was found to significantly inhibit the uptake of 1-methyl-4-phenylpyridinium mediated by OCT1 or MATE1. In contrast, knockdown of IRIP by small hairpin RNA (shRNA) increased the transporter activities *in vitro*. IRIP overexpression decreased the membrane localization of transporter proteins without any changes in transcript levels in cells. By overexpressing IRIP in mouse liver *via* hydrodynamic tail vein injection, we demonstrated that increased IRIP expression could cause a significant reduction in hepatic accumulation of metformin ($P < 0.01$). In addition, we observed that the expression of IRIP was approximately half ($P < 0.01$) in *ob/ob* mice when compared to their lean littermates, with significant increases in hepatic Oct1 protein expression and metformin accumulation. In conclusion, IRIP negatively modulates the function of OCT1 and MATE1 in cells. Importantly, we provide *in vivo* evidence for such modulation that may cause an alteration in drug disposition. The regulation by IRIP on transporter activities likely occurs at a post-transcriptional level, and future studies are needed to characterize the exact mechanism.

Keywords

Ischemia/reperfusion-inducible protein; drug transporter; organic cation transporter; multidrug and toxin extrusion; hydrodynamic tail vein injection; obesity

INTRODUCTION

The critical role of xenobiotic transporter proteins in the disposition of clinically used drugs has been well appreciated^{1–5}. Among recognized examples, the organic cation transporter 1 (OCT1; SLC22A1) has been demonstrated by us as a key determinant of the therapeutic response to metformin^{6,7}, one of top prescription drugs in the United States. Despite their

*Correspondence to: Yan Shu, M.D., Ph.D., Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland at Baltimore, 20Penn Street, HSFII Room 555, Baltimore, MD 21201, USA, Phone: +01 410-706-7358, Fax: +01 410-706-7015, yshu@rx.umaryland.edu.

clinical significance, many xenobiotic transporters, particularly those uptake transporters including OCT1, are poorly characterized in their regulation. The recently identified ischemia/reperfusion-inducible protein (IRIP) regulates a variety of both uptake and efflux transporters including dopamine transporter (DAT), norepinephrine transporter (NET), serotonin transporter (SERT), OCT2, OCT3, organic anion transporter 1 (OAT1), and P-glycoprotein (P-gp) in cell culture systems^{8,9}.

IRIP represents the mammalian member of a protein family containing Sua5-YicO-YrdC domain⁸. Found in all sequenced genomes to date, the Sua5-YicO-YrdC family has been ranked in the top ten conserved hypothetical proteins to be considered as high priority for experimental study¹⁰. Attempts have been made to understand their functions in tRNA modification¹¹, ribosome biogenesis^{12,13} and normal growth^{14–17} in prokaryotes and yeast. However, the underlying mechanisms of these functions are still unknown. The only function reported for the higher eukaryotic IRIP homologues is the regulation of transporter activities^{8,9}. Nevertheless, it remains to determine whether IRIP has other targets and the regulation exists *in vivo* having an effect on drug disposition.

Disposition of certain cationic compounds is determined by cellular uptake *via* OCTs which are expressed in the basolateral membrane of hepatocytes and renal proximal tubular cells^{3,18,19}. More recently, the multidrug and toxin extrusion (MATE, SLC47A) proteins have been identified and characterized as H⁺/organic cation antiporters mediating the excretion of cationic compounds into bile and urine^{20–23}. The MATEs such as human MATE1 and MATE2K are located in the apical membrane of hepatocytes and/or renal proximal tubular cells. Notably, OCTs and MATEs share a broad spectrum of substrates and inhibitors²⁴. Thus, coordinated transcellular translocation by uptake *via* OCTs in concert with efflux *via* MATEs is believed to be an essential system for biliary and renal elimination of cationic drugs²⁵. Whereas human OCT2 and OCT3 has been demonstrated as a regulatory target for IRIP, it is unclear whether other transporters in the system, and hence the disposition of cationic drugs, are subject to IRIP regulation.

Drug disposition and response are affected by many pathophysiological conditions which may alter the activities of xenobiotic transporters in critical tissues such as liver and kidney. Among these conditions, obesity is particularly important as it is becoming a major worldwide health problem. Limited evidence suggests that drug transporter function could be altered due to obesity. For example, the expression of efflux transporters, such as multidrug resistance-associated proteins 2 (MRP2) and MRP4, are reported to be increased in livers of leptin deficient obese (*ob/ob*) mice²⁶. More *et al.* also found that there was increased expression of MRP3 and MRP4, but decreased expression of MRP1 in mouse livers of diet-induced diabetes²⁷. Because IRIP regulates multiple drug transporters and its expression is responsive to pathophysiological stimuli^{8,28}, it is intriguing to examine whether this protein contributes to any alteration of transporter function and drug disposition in obesity.

The present study is to investigate whether IRIP expression modulates the activities of OCT1 and MATE1, and hence *in vivo* disposition of their substrate metformin, a therapeutic drug for diabetes and other obesity-related syndromes. At first, the effect of IRIP expression on drug transport was determined in the human embryonic kidney 293 (HEK-293) cells expressing OCT1 or MATE1. We then measured metformin pharmacokinetics in the mice with hepatic overexpression of IRIP. Lastly, the hepatic expression of IRIP and metformin transporters, and metformin pharmacokinetics were compared between obese and lean mice.

EXPERIMENTAL SECTION

Materials

1-methyl-4-phenylpyridinium (MPP⁺), metformin, geneticin (G418) and puromycin were purchased from Sigma-Aldrich (St Louis, MO). ³H-MPP⁺ was purchased from PerkinElmer Inc. (Boston, Massachusetts). ¹⁴C-metformin was purchased from Moravsek Biochemicals Inc. (Brea, California). Lipofectamine 2000 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen Inc. (Carlsbad, California). All other reagents except those specifically described below were commercially available.

Expression Plasmids, Cell culture and Transfection

The plasmids for ectopic expression of mouse IRIP (mIrip), mouse Oct1 (mOct1), and mouse Mate1 (mMate1) (pCMV-SPORT6-mIRIP, pCMV-SPORT6-hMATE1, pCMV-SPORT6-mOct1, and pCMV-SPORT6-mMate1, respectively) were purchased from Thermo Scientific Co. (Lafayette, CO). The plasmid for the expression of human IRIP (pCMV-Tag3B-hIRIP) was a gift from Dr. Oleg Mirochnitchenko (Robert Wood Johnson Medical School, Piscataway, New Jersey). The plasmid for the expression of a functional EGFP-hOCT1 fusion protein was described previously ²⁹. The full length cDNA of mouse IRIP was amplified from pCMV-SPORT6-mIrip by primer pairs with BamH I and Xho I restriction enzyme sequences at 5' and 3' termini respectively. The PCR product was double digested by BamH I and Xho I, and then subcloned into the pLiver vector (pLIVETM, Mirus Bio LCC, WI) which is designed for high level, prolonged expression of transgenes in mouse livers. For human IRIP knockdown, several shRNAs were constructed by inserting potential sequences with BamH I and EcoR V sticky ends into the shRNA expression pGreen-puro vector containing the H1 promoter (System Bioscience, Mountain View, CA) with the same restriction enzyme sites. Knockdown efficiency by a shRNA was verified by real-time PCR, and two shRNAs (SH1 and SH8) causing more than 70% IRIP transcript decrease were selected for later experiments. shRNA1(SH1) sequence: sense, gatccAGACTTGGCTCAGATGTTTGTTCAAGAGACAAACATCTGAGCCAAGTGTttttg; and antisense, aattcAAAAAAGACTTGGCTCAGATGTTTGTCTCTTGAACAAACATCTGAGCCAAGTGTg. shRNA(SH8) sequence: sense, gatccCAGTTGTCCTTGGTTATTGATGGTTCAAGAGACCATCAATAACCAAGGACA ACTG tttttg; and antisense, aattcAAAAAACAGTTGTCCTTGGTTATTGATGGTTCTCTTGAACCATCAATAACCAAGGA CAACTGg. (The underlined lower-case letters represent sticky ends; the underlined upper-case letters represent shRNA loop.)

HEK-293 cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA). Cells were grown in DMEM, supplemented with 10% FBS, 4.5 mM glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml), and were maintained in 75-cm² plastic flasks in 5% CO₂ at 37°C. HEK-293 cells were plated in 24-well bio-coated plates (2 cm²/well) with 25 × 10⁴/well for 18–24 hours (h) before transfection. To study the effect of IRIP expression on OCT1 function, the EGFP-OCT1 plasmid was transfected into cells along with an empty vector, pCMV-Tag3B-hIRIP, pCMV-SPORT6-mIrip, scrambled shRNA, hIRIP SH1 or SH8, using Lipofectamine 2000 according to manufacturer's instruction. Images were dynamically taken under a microscope since 6 h after transfection. ³H-MPP⁺ uptake experiments were conducted as below. To study the effect of IRIP expression on MATE1 function, the same procedure was performed as described above, except the use of MATE1 plasmids instead of OCT1 ones.

Drug Uptake in Cells

All uptake experiments were conducted on monolayer cultures in 24-well plates at 37°C. For studying the drug uptake mediated by MATE1, a K⁺ based buffer was used, which consisted of 140 mM KCl, 0.4 mM KH₂PO₄, 0.8 mM MgSO₄, 1.0 mM CaCl₂, 25 mM glucose, and 10 mM HEPES, pH 7.4. Before transport assays, cells were washed with pre-warmed K⁺ based buffer and then incubated in the K⁺ based buffer containing 30 mM NH₄Cl for 10 min at 37°C, and thereafter incubated in the uptake buffer (NH₄Cl-free) for 5 min. Transport assays were started by replacing the substrate-free uptake buffer with that containing a ³H-labeled MPP⁺ substrate for 5 min at room temperature. The assays were stopped by addition of ice-cold substrate-free uptake buffer, and the cells were washed 3 times. To assess OCT1 uptake function, KRH buffer (125 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM HEPES, 5.6 mM glucose, pH 7.4) was used. The cells were washed once with pre-warmed uptake buffer. After incubation with uptake buffer containing different concentrations of MPP⁺ with a ³H tracer for different time, the uptake was halted by removing the uptake buffer and washing the cells with ice-cold uptake buffer 3 times. Cells were solubilized in 0.1N sodium hydroxide (NaOH). The plate was shaken for 30 minutes and added 0.1N hydrochloric acid (HCl) to neutralize the buffer. The cell lysate was then transferred to scintillation tubes containing 3 ml biodegradable counting cocktail buffer (Fisher Scientific Inc., Pittsburgh, PA). Radioactivity was determined by a multi-purpose scintillation counter (Beckman LS6500 Counter, Brea, CA). Protein concentrations were measured using a BCA protein assay kit (Bio-Rad, Hercules, CA) to normalize the radioactivity.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from HEK-293 cells or the homogenates of mouse livers using Trizol and phenol-chloroform extraction, and 2 µg of total RNA was reversely transcribed to cDNA using a high capability reverse transcript kit (Roche Applied Science, Indianapolis, IN). The mRNA levels of genes of interest were determined by qRT-PCR on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster city, CA). All primers used in the study were designed with the software Oligo 5.0 and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). A total volume of 12.5 µl reaction mixture consisted of 1 µl of cDNA template, 6.25 µl SYBR green PCR master Mix (SABiosciences, Frederick, MD), and 1.0 µl 2.5 µM primer mixture. The PCR amplification was performed with 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the internal control. The fluorescent signals were collected during the extension phase, cycle threshold (Ct) values of the sample calculated, and gene transcript levels analyzed by the 2^{-ΔΔCt} method³⁰

Western Blotting

Liver tissues were minced with scissors in ice-cold Tris-sucrose buffer (100 mM Tris, 250 mM sucrose, pH 7.6) in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Diagnostics, Mannheim, Germany) and then homogenized with PowerGen125 (Fisher Scientific Co. Pittsburgh, PA) operating at 8000 rpm³¹. A membrane-enriched microsomal pellet was obtained from the post-nuclear supernatant after a 100,000g ultracentrifugation for 1 h at 4°C. The pellet was re-suspended in HEPES-sucrose buffer (10 mM HEPES, 300 mM sucrose, pH 7.5, protease inhibitors). To prepare membrane fractions from cultured HEK-293 cells, the cells were disrupted by sonication in hypotonic buffer (1 mM EDTA, 5 mM sodium/potassium phosphate; pH 7.0). The pellets were re-suspended in Tris buffer (50 mM; pH 7.4) after ultracentrifugation of 100,000g at 4°C for 45 min³². The protein concentration was determined according to a BCA protein assay kit (Bio-Rad Co. Hercules, CA), and samples were stored at -80°C until use. The membrane fractions were mixed with the sample-loading buffer (Bio-Rad, Hercules, CA)

and denatured at 37°C for 20 min, and then adequate amounts of protein (75 µg total membrane) were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA). Tris/Glycine running buffer was used for electrophoresis, and the gel was gently washed in transfer buffer (24.8 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 5 minutes. Proteins in the gel were stained by Coomassie blue dye to ensure protein integrity, quality, and equally loading. Proteins were transferred to nitrocellulose membrane in the presence of transfer buffer in Criterion Plate (Bio-Rad Co., Hercules, CA). The membranes were incubated in the blocking solution containing 5% milk for 1 hour at room temperature, and then in the presence of 1:200 (OCT1 antibody sc-133866, Santa Cruz, CA) or 1:500 (EGFP antibody, Cell Signaling, Danvers, MA) primary antibodies overnight at 4°C. The membranes were washed 3 times using TBST buffer (25 mM Tris base, 137 mM NaCl, 3 mM KCl, 0.1% Tween 20) for 5 min/per time and then incubated in the blocking solution with 1:5000 anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling, Danvers, MA). Finally, the proteins were detected using the western lightning-ECL (Enhanced Chemiluminescence Reagent) (PerkinElmer, Waltham, MA). The densitometry for immunoblots was conducted using Alphaview SA version 3.4.0 (ProteinSimple, Santa Clara, CA).

Animals

All procedures were carried out in accordance with NIH guidelines for animal experimentation, and the experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the School of Pharmacy, University of Maryland at Baltimore. C57BL mice were bought from the Jackson Laboratory (Bar Harbor, ME) and were housed under controlled conditions ($21 \pm 2^\circ\text{C}$, humidity $60 \pm 10\%$ and 12h/12h dark/light cycle) and had free access to food and water.

Hydrodynamic Tail Vein Injection (HTV)

The expression plasmid encoding mouse IRIP (*pLiver-mIRIP*) was constructed as described above. HTV was performed as described previously^{33, 34}. In brief, injection was performed with a 27-gauge, 0.45-inch needle. The pLiver-mIrip plasmids (90 µg/ml) were dissolved in TransIT-QR Hydrodynamic Delivery Solution (Mirus Bio LLC., Pittsburgh, PA). Total volume needed per mouse (in ml) was calculated as mouse weight (g)/10 plus 0.1 ml of delivery solution which was to compensate the volume remaining in the syringe and needle after injection. The solution was injected into the mice *via* the tail vein in 4–7 seconds. The expression of mIRIP in different tissues, including liver, kidney, brain and heart, were assessed at different times after HTV. Stable hepatic transgene expression was achieved for at least 3 months, and the later experiments were conducted on the mice 2 weeks after HTV. The *pLive* containing a cDNA of enhanced green fluorescent protein (*pLive-EGFP*) was also used for assessment of the gene delivery efficiency by HTV. Two weeks after the injection of *pLive-EGFP* plasmids, the mice were euthanized, and the livers were divided into three sections composed of median, left, and right lobes. The liver pieces were collected from each lobe and embedded in Tissue-Tek OCT compound (Electron Microscopy Sciences, Hatfield, PA) and was fast frozen in liquid nitrogen. Cryo-section slides were stained by DAPI and observed under a fluorescence microscope.

Hepatic Metformin Accumulation

Twelve-week old male mice were fasted for 4 hours and then injected intraperitoneally with 15 mg/kg metformin (containing 1/20 ¹⁴C-metformin). The mice were euthanized 30 min later and the liver tissues were isolated, gently washed, weighed and submerged in PBS, which were further homogenized completely. The homogenized tissues were centrifuged at

15,000 rpm for 10 minutes and the radioactivity in the supernatant was counted at a multiple-purpose scintillation counter (Beckman LS6500 Counter, Brea, CA).

Metformin Pharmacokinetics Study

Twelve-week old male mice were fasted for 4 hours and then injected intraperitoneally with 15 mg/kg metformin (containing 1/20 ^{14}C -metformin). Blood samples were collected by tail bleeding at 0, 8, 15, 30, 45, 60, 90, and 120 minutes after injection. Blood samples were centrifuged at 8000 rpm for 10 min at 4°C and 6 μl of plasma were added into 2 ml scintillation buffer. The radioactivity in the plasma was counted at a multiple-purpose scintillation counter (Beckman LS6500 Counter, Brea, CA).

Statistics and Data Analysis

WinNonlin (version 5.2.1, Pharsight Corporation, Mountain View, CA) was used to analyze the plasma concentration-time profiles of metformin after the intraperitoneal administration in mice. The area under the blood concentration-time curve from time 0 to infinity (AUC_{∞}) were calculated by the nonlinear least-squares method. The AUC until 120 min (AUC_{0-120}) was determined by the trapezoidal rule.

Unless indicated, the data are presented as mean $\pm$ standard deviation (SD). Two tailed *Student's t*-test was applied to analyze data, when appropriate. For multiple comparison tests, *ANOVA* was used followed by *Dunnett's* test. A *P* value of < 0.05 was considered statistically significant.

RESULTS

IRIP overexpression inhibited the activities of OCT1 and MAE1 in HEK-293 cells

IRIP has been previously reported to negatively regulate several transporter proteins in cell cultures^{8,9}. Here we at first sought to determine whether IRIP regulated the activities of human OCT1 and MATE1 as additional targets. HEK293 cells were cotransfected with a transporter expression plasmid and either an empty or IRIP expression plasmid vector. MPP^+ uptake for 5 min was performed to assess the activities of OCT1 and MATE1. The overexpression of human IRIP (hIRIP) and mouse IRIP (mIRIP) significantly reduced the activity of OCT1 and MATE1 by 35% and 34%, and 41% and 39%, respectively (Figure 1). hIRIP overexpression also suppressed the MPP^+ uptake mediated by mouse MATE1 (data not shown). The data suggested a conserved function among mammalian IRIPs. By using RT-PCR, however, we did not detect any differences in the levels of mRNA for either OCT1 or MATE1 due to IRIP co-expression, suggesting an underlying post-transcriptional mechanism. We dynamically examined the effect of hIRIP expression on EGFP-hOCT1 protein expression in HEK293 cells. At 12, 24, and 36 hours after co-transfection, images were taken under a fluorescence microscope. We found that the expression of EGFP-hOCT1 was suppressed by hIRIP co-expression over this period (Figure 2A). We then isolated cellular membrane fraction from the transfected HEK293 cells. The membrane expression of EGFP-hOCT1 protein was significantly decreased in the cells expressing hIRIP than those expressing the control empty vector (Figure 2B). In contrast to this, the overall integral membrane protein levels were not altered by IRIP overexpression (Figure 2C). We noticed that in contrast to the dramatic suppression of EGFP-hOCT1 protein expression by IRIP overexpression (Fig. 2), MPP^+ uptake mediated by hOCT1 was only reduced by 35% by IRIP overexpression (Fig. 1A). In the overexpression system, a large amount of fusion protein remained in the cytoplasm. The drug uptake might only reflect the functional OCT1 proteins that were successfully trafficked to the plasma membrane. Thus, one explanation is that the extent of function suppression might not be necessarily similar to that of suppression on overall protein expression. The immunoblotting results suggested that the inhibitory

effect of IRIP expression on transporter function was due to a decreased expression of transporter proteins in cell membrane.

IRIP knockdown by shRNA increased the activity of OCT1 in HEK293 cells

To further validate if IRIP expression was required for the full function of a drug transporter protein, we examined the effect of IRIP knockdown on the activity of OCT1. Six hIRIP shRNA sequences were designed using online siRNA design software (www.genelink.com) and inserted into a modified shRNA expression vector (pGreenPuroTM-shRNA). HEK293 cells endogenously expressed a significant level of IRIP. By using RT-PCR, we confirmed that two of the shRNA constructs (SH1 and SH8) efficiently suppressed the transcript levels of IRIP in HEK293 cells, with more than 65% knockdown effects. HEK293 cells were transfected with SH1, SH8 or their combination (SH18). Compared to the cells expressing the scrambled shRNA, those transiently expressing SH1 and/or SH8 had significantly lower levels of IRIP mRNA (Figure 3A). No growth or morphological changes were observed in these transfected cells. We then performed MPP⁺ uptake studies in the HEK293-SH18 cells with transient expression of hOCT1, hIRIP, or control plasmids. As expected, while MPP⁺ uptake was decreased by IRIP overexpression, IRIP knockdown significantly increased OCT1 function (Figure 3B). The maximal effect by IRIP knockdown was seen 12 hours after OCT1 plasmid transfection. Notably, IRIP knockdown in HEK293 cells also increased the basal uptake of MPP⁺ that might not be ascribed to one single transporter protein (data not shown). We also conducted similar experiments to examine the effect of IRIP knockdown on MATE1 activity. Although we did see a trend that IRIP knockdown increased MPP⁺ uptake mediated by MATE1, the increase hardly reached a statistical significance (data not shown). It is possible that IRIP has a weaker regulatory effect on MATE1 function.

Hepatic IRIP overexpression was associated with decreased hepatic metformin accumulation in mice

To explore IRIP's regulatory effect on drug disposition *in vivo*, the hydrodynamic tail vein (HTV) injection was used to overexpress *IRIP* in mouse liver. Mouse *IRIP* cDNA was subcloned into the *pLIVE*TM vector containing the liver-specific mouse albumin promoter and an enhancer. A total of 90 µg of plasmid DNA was injected into the tail vein of 6-week-old C57BL/6 mice in a delivery volume equivalent to 10% of the body mass of each mouse within 4 – 7 seconds. We firstly visualized the hepatic transfection efficiency by this method with the plasmid vector of GFP gene. Four days after HTV injection, GFP fluorescence in cryo-sections of mouse liver was examined. Apparently, the GFP transgene was highly expressed in the liver *via* HTV injection (Figure 4A). The hepatic overexpression of *IRIP* *via* HTV was confirmed by RT-PCR. At 4 days after HTV injection, the mRNA level of *mIRIP* was highly elevated in the mice received the *mIRIP* plasmids by over 16 folds when compared to those received the control plasmids (Figure 4B). The elevated expression was sustained for at least 3 months (data not shown). As the physical hepatic injury after HTV injection was found around portal vein during first several days, the mice at 2 weeks after HTV injection was used for further drug disposition study. The blood chemistry, including the enzymatic levels of liver injury (AST and ALT), did not show any significant differences between the *mIRIP*-overexpression group compared with the group received control plasmids at 3 weeks after HTV injection (data not shown). Interestingly, the *mIRIP*-overexpression group gained 10% more body weight than the control group (*mIRIP* vs. empty plasmids, 25.5 ± 1.0 vs. 23.7 ± 1.6; *P* = 0.03) at 3 weeks after HTV injection.

By studying metformin, we sought to determine if IRIP expression would affect *in vivo* drug disposition *via* its regulation on transporter function. The cloned metformin transporters include OCTs and MATEs^{6, 7, 35–38} which are interestingly subject to IRIP regulation *in*

vitro (Figures 1–3)⁸. The mice were intraperitoneally administrated with metformin (15 mg/kg) at 3 weeks after HTV injection. Metformin accumulation in the liver was remarkably decreased in the *mIRIP*-overexpression group compared with the group received empty plasmids (*mIRIP* vs. empty plasmids, 362 ± 64.7 vs. 975 ± 125 ng/g, $P = 0.002$) (Figure 4C). The data suggested that *IRIP* expression played an important role in regulation of metformin tissue distribution *in vivo*. However, we did not observe any differences in the overall pharmacokinetic profile of metformin between the two groups (Figure 4D).

The obese *OB/OB* mice had reduced *IRIP* expression and increased metformin accumulation in the liver

To further understand the function of *IRIP* *in vivo* on drug disposition, we examined the expression of *IRIP* and metformin pharmacokinetics in the animal model of obesity which may be treated with metformin. The hepatic mRNA level of *IRIP* in the *ob/ob* mice was significantly decreased by 48% compared to their lean littermates (Figure 5A). Consistent hepatic *IRIP* protein reduction was also detected in the *ob/ob* mice, and the mice fed a high-fat diet showed similar results (data not shown). Interestingly, the membrane protein levels of Oct1, a major metformin transporter in mouse liver, were found to be significantly higher in the *ob/ob* mice than those in the lean mice (Figure 5B), which was in line with a negative regulation by *IRIP* on OCT1 protein above *in vitro* (Figure 2). Notably, the hepatic mRNA levels of several metformin transporters, including Oct1 (Figure 5C), Oct2, Oct3, and Mate1, were either significantly reduced (Oct1 and Oct3) or little altered (Oct2 and Mate1) in the *ob/ob* mice.

The obese and lean mice were intraperitoneally administrated with metformin (15 mg/kg). In line with the decreased expression of *IRIP* and increased expression of Oct1, hepatic metformin accumulation was remarkably elevated in the *ob/ob* mice compared with the lean mice (Figure 5D). We also observed a significant difference in overall pharmacokinetic profile between the *ob/ob* mice and their lean littermates (Figure 5E, Table 1). The maximal blood concentration ($C_{\max}$) was significantly lower in the *ob/ob* mice than that in the lean mice. The clearance of metformin was slower in the former than the latter.

DISCUSSION

IRIP represents the mammalian member of the Sua5-YicO-YrdC protein family that has been studied for two decades^{8–17}. These previous studies were almost conducted in prokaryotes and yeast. *IRIP* homologues are highly conserved in amino acid sequences, suggesting an important function during evolution. However, the function is still unclear in prokaryotes, yeast, and higher eukaryotes, including humans and other mammals. The present work both *in vitro* and *in vivo*, along with two recent studies in cell cultures^{8,9}, shed a light on *IRIP* function in mammals by demonstrating that the protein can modulate the activities of the transporters of xenobiotics and neurotransmitters.

The regulatory effect of *IRIP* on transporter function was found as serendipity. Jiang *et al.* isolated *IRIP* cDNA in an attempt to understand the genes involved in ischemia/reperfusion injury by using differential display analysis. The protein was then found to interact with RS1, which had been characterized previously to negatively regulate the activities of various transporters including 3 Na(+)-D-glucose cotransporter 1 (SGLT1), OCT2, and concentrative nucleoside transporters (CNTs)^{8,39–45}. The role of *IRIP* in regulating transporter function was subsequently investigated. While knockdown of *IRIP* expression increased the uptake of the organic cation MPP⁺ into HeLa cells, *IRIP* overexpression caused the inhibition of endogenous as well as exogenously expressed uptake transporters, including OCT2, OCT3, OAT1, DAT, NET and SERT. The same group later reported that, in addition to uptake transporters, *IRIP* also negatively modulated the activity of the efflux transporter P-

glycoprotein (P-gp). It seemed that IRIP did not influence P-gp biosynthesis but affect its processing and promote its degradation by interrupting the interaction of P-gp with COP- α , one of the coat proteins I vesicle (COPI) subunits involving in intra-Golgi trafficking of membrane proteins. In the present study, we further identified OCT1 and MATE1 as the targets of IRIP regulation (Figure 1). Multiple small cationic drugs (e.g., metformin) have been identified as substrates for both the basolateral OCTs as well as apical MATE transporters. These transporters likely work in concert in the disposition of small cationic drugs in the liver and kidney. IRIP is highly expressed in the liver and kidney⁸. Therefore, our *in vitro* findings particularly implicate that IRIP is involved in the disposition of small cationic drugs by regulating their uptake into and/or elimination from these critical organ tissues of detoxification (Figure 6). It seemed that IRIP did not affect the transcript levels of OCT1 and MATE1 in the cell culture systems *in vitro* with overexpressed transporters. However, the transporter protein levels, in particular those at the membrane, were downregulated by IRIP expression (Figure 2). It should be noted that RS1, which interacts with IRIP⁸, regulates the activity of membrane transporters *via* endocytotic and exocytotic pathways^{43–45}. Although the activity of human OCT2 is subject to regulation by both RS1 and IRIP, it is not known whether other transporters regulated by IRIP are regulatory targets of RS1, and *vice versa*. Whereas the exact mechanism of IRIP regulation on transporter activities is our on-going effort, current results implicate one at post-transcriptional levels.

For the first time, we demonstrated that IRIP indeed regulated drug disposition *in vivo*. By using hydrodynamic tail vein (HTV) injection, we overexpressed IRIP in mouse livers, and measured hepatic accumulation of metformin after the mice were treated with metformin. There was a remarkable decrease of metformin accumulation in the liver with hepatic IRIP overexpression (Figure 4). Metformin, which is highly hydrophilic and positively-charged under physiological conditions due to the strongly basic character of its polar guanidine moiety, relies on transporter proteins to cross cellular membrane⁶. The drug is eliminated from the body mainly by the kidney without significant metabolism⁴⁶. The characterized transporters for metformin include OCT1–3, MATE1–2, and PMAT^{47, 48}. Except PMAT which remains to be studied, these metformin transporters are subject to IRIP regulation. Mice highly express Oct1 in the liver, with minor or moderate expression of other metformin transporters^{35, 49}. Therefore, the decreased metformin accumulation in mouse livers is very likely due to the inhibition of metformin uptake transporters, particularly Oct1, by IRIP overexpression. In contrast, the inhibition of metformin efflux transporters such as apical MATE1 by IRIP overexpression is expected to increase cellular metformin accumulation. Our data thus suggest that uptake may be more rate-limiting than elimination in metformin accumulation in the liver, at least in the time frame studied (Figure 6).

The overall pharmacokinetics profile did not show any changes in the mice with hepatic IRIP overexpression. Previously, the Oct1 knockout mice (*Oct1*^{–/–}) had a similar pharmacokinetic profile to that of wild-type mice⁷. It is therefore possible that changes in transporter activities in the liver only may be insufficient to cause a systemic change in the disposition of metformin, which is mainly eliminated from the kidney⁴⁶. IRIP is ubiquitously expressed with a high expression in the kidney. It is extremely intriguing to examine how a global change in IRIP expression may affect transporter activities and drug disposition. This should be achieved by generating IRIP knockout and/or transgenic mice.

Metformin is a first-line drug for type 2 diabetes and a potential therapy for fatty liver disease, metabolic syndrome, polycystic ovary syndrome, and cancer that are related to obesity^{50–54}. It was reported previously that hepatic cytochrome P-450 (CYP), conjugation, and antioxidant enzymes in *ob/ob* mice were altered⁵⁵, which implicates a complicated situation for drug therapy of obesity-related syndromes. However, few studies on the alteration of transporters in a fatty liver are reported. In the present study, we found the

hepatic expression of IRIP was significantly decreased in *ob/ob* mice compared to lean mice (Figure 5). Interestingly, this was accompanied with an increased Oct1 protein expression in the membrane fraction of liver tissues and an enhanced hepatic metformin accumulation. The data is in line with the effect of hepatic IRIP overexpression on metformin accumulation (Figure 4), indicating a negative modulation by IRIP on transporter function *in vivo*. Notably, the mRNA level of hepatic *Oct1* in *ob/ob* mice was actually lower than that in lean mice. Although the exact details are subject to future intensive research, this may be reconciled by a feedback mechanism. The decreased expression of certain genes such as IRIP, which affect the expression of transporters at post-transcriptional levels such as reduced degradation by proteasome (e.g., P-glycoprotein⁹), may increase functional transporter protein amounts. As such, by a feedback mechanism, less transporter gene transcripts are required to maintain the homeostasis *via* the transporter proteins. The pharmacokinetic profile of metformin was significantly altered in *ob/ob* mice. Whereas this may reflect pathophysiological changes due to obesity, altered transporter function *via* IRIP regulation could be a contributing mechanism. For example, the increased uptake of metformin into the liver due to the enhanced transporter function may cause the slower absorption phase and lower C_{max} in *ob/ob* mice. Liver is a major target organ in metformin therapy. The enhanced metformin accumulation in the liver of the obesity animal model has significant clinical implication. Clinical studies indicate that the therapeutic glucose-lowering effect by metformin is hardly observed in healthy subjects^{56, 57}. Previously we found that metformin did not lower fasting glucose level in normal mice unless fed on a high-fat diet for 9 weeks⁶. Further studies on the effect of obesity or diabetes on IRIP expression and transporter function are warranted.

In conclusion, we further identified that OCT1 and MATE1 are among the transporters subject to IRIP regulation. For the first time, we demonstrated that IRIP in mouse livers could regulate drug disposition *in vivo*, possibly *via* its negative modulation on transporter activities. Supportive evidence was obtained from an obesity mouse model that the obese mice exhibited a decreased IRIP expression, increased Oct1 expression and metformin accumulation in the liver when compared to lean mice. The regulation by IRIP on transporter activities likely occurs at a post-transcriptional level. However, future studies are needed to characterize the exact mechanism.

Acknowledgments

The present study was supported by the National Institute of General Medical Sciences of the National Institutes of Health (NIH) under Award R01GM099742 and by the US Food and Drug Administration (FDA) under Award U01FD004320. Qing Li received research support from National Natural Science Foundation (NNSF) of China (81001445). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH, FDA and NNSF.

References

1. Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, Ishikawa T, Keppler D, Kim RB, Lee CA, Niemi M, Polli JW, Sugiyama Y, Swaan PW, Ware JA, Wright SH, Yee SW, Zamek-Gliszczynski MJ, Zhang L. Membrane transporters in drug development. *Nat Rev Drug Discov.* 2010; 9(3):215–36. [PubMed: 20190787]
2. Hagenbuch B, Gui C. Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. *Xenobiotica.* 2008; 38(7–8):778–801. [PubMed: 18668430]
3. Koepsell H, Lips K, Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharm Res.* 2007; 24(7):1227–51. [PubMed: 17473959]

4. VanWert AL, Gionfriddo MR, Sweet DH. Organic anion transporters: discovery, pharmacology, regulation and roles in pathophysiology. *Biopharm Drug Dispos.* 2010; 31(1):1–71. [PubMed: 19953504]
5. Lagas JS, Vlaming ML, Schinkel AH. Pharmacokinetic assessment of multiple ATP-binding cassette transporters: the power of combination knockout mice. *Mol Interv.* 2009; 9(3):136–45. [PubMed: 19592674]
6. Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, Ianculescu AG, Yue L, Lo JC, Burchard EG, Brett CM, Giacomini KM. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest.* 2007; 117(5):1422–31. [PubMed: 17476361]
7. Shu Y, Brown C, Castro RA, Shi RJ, Lin ET, Owen RP, Sheardown SA, Yue L, Burchard EG, Brett CM, Giacomini KM. Effect of genetic variation in the organic cation transporter 1, OCT1, on metformin pharmacokinetics. *Clin Pharmacol Ther.* 2008; 83(2):273–80. [PubMed: 17609683]
8. Jiang W, Prokopenko O, Wong L, Inouye M, Mirochnitchenko O. IRIP, a new ischemia/reperfusion-inducible protein that participates in the regulation of transporter activity. *Mol Cell Biol.* 2005; 25(15):6496–508. [PubMed: 16024787]
9. Prokopenko O, Mirochnitchenko O. Ischemia-reperfusion-inducible protein modulates cell sensitivity to anticancer drugs by regulating activity of efflux transporter. *Am J Physiol Cell Physiol.* 2009; 296(5):C1086–97. [PubMed: 19279227]
10. Galperin MY, Koonin EV. ‘Conserved hypothetical’ proteins: prioritization of targets for experimental study. *Nucleic Acids Res.* 2004; 32(18):5452–63. [PubMed: 15479782]
11. Teplova M, Tereshko V, Sanishvili R, Joachimiak A, Bushueva T, Anderson WF, Egli M. The structure of the yrdC gene product from *Escherichia coli* reveals a new fold and suggests a role in RNA binding. *Protein Sci.* 2000; 9(12):2557–66. [PubMed: 11206077]
12. El Yacoubi B, Lyons B, Cruz Y, Reddy R, Nordin B, Agnelli F, Williamson JR, Schimmel P, Swairjo MA, de Crecy-Lagard V. The universal YrdC/Sua5 family is required for the formation of threonylcarbamoyladenine in tRNA. *Nucleic Acids Res.* 2009; 37(9):2894–909. [PubMed: 19287007]
13. Kaczanowska M, Ryden-Aulin M. The YrdC protein--a putative ribosome maturation factor. *Biochim Biophys Acta.* 2005; 1727(2):87–96. [PubMed: 15716138]
14. Agari Y, Sato S, Wakamatsu T, Bessho Y, Ebihara A, Yokoyama S, Kuramitsu S, Shinkai A. X-ray crystal structure of a hypothetical Sua5 protein from *Sulfolobus tokodaii* strain 7. *Proteins.* 2008; 70(3):1108–11. [PubMed: 18004774]
15. Lin CA, Ellis SR, True HL. The Sua5 protein is essential for normal translational regulation in yeast. *Mol Cell Biol.* 2010; 30(1):354–63. [PubMed: 19884342]
16. Meng FL, Chen XF, Hu Y, Tang HB, Dang W, Zhou JQ. Sua5p is required for telomere recombination in *Saccharomyces cerevisiae*. *Cell Res.* 2010; 20(4):495–8. [PubMed: 20309016]
17. Na JG, Pinto I, Hampsey M. Isolation and characterization of SUA5, a novel gene required for normal growth in *Saccharomyces cerevisiae*. *Genetics.* 1992; 131(4):791–801. [PubMed: 1325384]
18. Nies AT, Koepsell H, Winter S, Burk O, Klein K, Kerb R, Zanger UM, Keppler D, Schwab M, Schaeffeler E. Expression of organic cation transporters OCT1 (SLC22A1) and OCT3 (SLC22A3) is affected by genetic factors and cholestasis in human liver. *Hepatology.* 2009; 50(4):1227–40. [PubMed: 19591196]
19. Zolk O, Solbach TF, König J, Fromm MF. Structural determinants of inhibitor interaction with the human organic cation transporter OCT2 (SLC22A2). *Naunyn Schmiedeberg Arch Pharmacol.* 2009; 379(4):337–48. [PubMed: 19002438]
20. Otsuka M, Matsumoto T, Morimoto R, Arioka S, Omote H, Moriyama Y. A human transporter protein that mediates the final excretion step for toxic organic cations. *Proc Natl Acad Sci U S A.* 2005; 102(50):17923–8. [PubMed: 16330770]
21. Masuda S, Terada T, Yonezawa A, Tanihara Y, Kishimoto K, Katsura T, Ogawa O, Inui K. Identification and functional characterization of a new human kidney-specific H⁺/organic cation antiporter, kidney-specific multidrug and toxin extrusion 2. *J Am Soc Nephrol.* 2006; 17(8):2127–35. [PubMed: 16807400]

22. Omote H, Hiasa M, Matsumoto T, Otsuka M, Moriyama Y. The MATE proteins as fundamental transporters of metabolic and xenobiotic organic cations. *Trends Pharmacol Sci.* 2006; 27(11): 587–93. [PubMed: 16996621]
23. Tanihara Y, Masuda S, Sato T, Katsura T, Ogawa O, Inui K. Substrate specificity of MATE1 and MATE2-K, human multidrug and toxin extrusions/H(+)-organic cation antiporters. *Biochem Pharmacol.* 2007; 74(2):359–71. [PubMed: 17509534]
24. Damme K, Nies AT, Schaeffeler E, Schwab M. Mammalian MATE (SLC47A) transport proteins: impact on efflux of endogenous substrates and xenobiotics. *Drug Metab Rev.* 2011; 43(4):499–523. [PubMed: 21923552]
25. Matsuzaki T, Morisaki T, Sugimoto W, Yokoo K, Sato D, Nonoguchi H, Tomita K, Terada T, Inui K, Hamada A, Saito H. Altered pharmacokinetics of cationic drugs caused by down-regulation of renal rat organic cation transporter 2 (Slc22a2) and rat multidrug and toxin extrusion 1 (Slc47a1) in ischemia/reperfusion-induced acute kidney injury. *Drug Metab Dispos.* 2008; 36(4):649–54. [PubMed: 18180268]
26. Cheng Q, Aleksunes LM, Manautou JE, Cherrington NJ, Scheffer GL, Yamasaki H, Slitt AL. Drug-metabolizing enzyme and transporter expression in a mouse model of diabetes and obesity. *Mol Pharm.* 2008; 5(1):77–91. [PubMed: 18189363]
27. More VR, Slitt AL. Alteration of hepatic but not renal transporter expression in diet-induced obese mice. *Drug Metab Dispos.* 2011; 39(6):992–9. [PubMed: 21430232]
28. Sauvant C, Schneider R, Holzinger H, Renker S, Wanner C, Gekle M. Implementation of an in vitro model system for investigation of reperfusion damage after renal ischemia. *Cell Physiol Biochem.* 2009; 24(5–6):567–76. [PubMed: 19910697]
29. Shu Y, Leabman MK, Feng B, Mangravite LM, Huang CC, Stryke D, Kawamoto M, Johns SJ, DeYoung J, Carlson E, Ferrin TE, Herskowitz I, Giacomini KM. Evolutionary conservation predicts function of variants of the human organic cation transporter, OCT1. *Proc Natl Acad Sci U S A.* 2003; 100(10):5902–7. [PubMed: 12719534]
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001; 25(4):402–8. [PubMed: 11846609]
31. Denk GU, Soroka CJ, Mennone A, Koepsell H, Beuers U, Boyer JL. Down-regulation of the organic cation transporter 1 of rat liver in obstructive cholestasis. *Hepatology.* 2004; 39(5):1382–9. [PubMed: 15122767]
32. Cui Y, Konig J, Buchholz JK, Spring H, Leier I, Keppler D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Mol Pharmacol.* 1999; 55(5):929–37. [PubMed: 10220572]
33. Sawyer GJ, Rela M, Davenport M, Whitehorne M, Zhang X, Fabre JW. Hydrodynamic gene delivery to the liver: theoretical and practical issues for clinical application. *Curr Gene Ther.* 2009; 9(2):128–35. [PubMed: 19355870]
34. Wooddell CI, Reppen T, Wolff JA, Herweijer H. Sustained liver-specific transgene expression from the albumin promoter in mice following hydrodynamic plasmid DNA delivery. *J Gene Med.* 2008; 10(5):551–63. [PubMed: 18330848]
35. Wang DS, Jonker JW, Kato Y, Kusuhara H, Schinkel AH, Sugiyama Y. Involvement of organic cation transporter 1 in hepatic and intestinal distribution of metformin. *J Pharmacol Exp Ther.* 2002; 302(2):510–5. [PubMed: 12130709]
36. Li Q, Peng X, Yang H, Wang H, Shu Y. Deficiency of multidrug and toxin extrusion 1 enhances renal accumulation of paraquat and deteriorates kidney injury in mice. *Mol Pharm.* 2011; 8(6): 2476–83. [PubMed: 21991918]
37. Tsuda M, Terada T, Mizuno T, Katsura T, Shimakura J, Inui K. Targeted disruption of the multidrug and toxin extrusion 1 (mate1) gene in mice reduces renal secretion of metformin. *Mol Pharmacol.* 2009; 75(6):1280–6. [PubMed: 19332510]
38. Chen L, Pawlikowski B, Schlessinger A, More SS, Stryke D, Johns SJ, Portman MA, Chen E, Ferrin TE, Sali A, Giacomini KM. Role of organic cation transporter 3 (SLC22A3) and its missense variants in the pharmacologic action of metformin. *Pharmacogenet Genomics.* 2010; 20(11):687–99. [PubMed: 20859243]

39. Reinhardt J, Veyhl M, Wagner K, Gambaryan S, Dekel C, Akhoundova A, Korn T, Koepsell H. Cloning and characterization of the transport modifier RS1 from rabbit which was previously assumed to be specific for Na⁺-D-glucose cotransport. *Biochim Biophys Acta*. 1999; 1417(1): 131–43. [PubMed: 10076042]
40. Veyhl M, Keller T, Gorboulev V, Vernaleken A, Koepsell H. RS1 (RSC1A1) regulates the exocytotic pathway of Na⁺-D-glucose cotransporter SGLT1. *Am J Physiol Renal Physiol*. 2006; 291(6):F1213–23. [PubMed: 16788146]
41. Veyhl M, Wagner CA, Gorboulev V, Schmitt BM, Lang F, Koepsell H. Downregulation of the Na⁺-D-glucose cotransporter SGLT1 by protein RS1 (RSC1A1) is dependent on dynamin and protein kinase C. *J Membr Biol*. 2003; 196(1):71–81. [PubMed: 14724758]
42. Errasti-Murugarren E, Fernandez-Calotti P, Veyhl-Wichmann M, Diepold M, Pinilla-Macua I, Perez-Torras S, Kipp H, Koepsell H, Pastor-Anglada M. Role of the transporter regulator protein (RS1) in the modulation of concentrative nucleoside transporters (CNTs) in epithelia. *Mol Pharmacol*. 2012; 82(1):59–67. [PubMed: 22492015]
43. Kroiss M, Leyerer M, Gorboulev V, Kuhlkamp T, Kipp H, Koepsell H. Transporter regulator RS1 (RSC1A1) coats the trans-Golgi network and migrates into the nucleus. *Am J Physiol Renal Physiol*. 2006; 291(6):F1201–12. [PubMed: 16788147]
44. Filatova A, Leyerer M, Gorboulev V, Chintalapati C, Reinders Y, Muller TD, Srinivasan A, Hubner S, Koepsell H. Novel shuttling domain in a regulator (RSC1A1) of transporter SGLT1 steers cell cycle-dependent nuclear location. *Traffic*. 2009; 10(11):1599–618. [PubMed: 19765263]
45. Korn T, Kuhlkamp T, Track C, Schatz I, Baumgarten K, Gorboulev V, Koepsell H. The plasma membrane-associated protein RS1 decreases transcription of the transporter SGLT1 in confluent LLC-PK1 cells. *J Biol Chem*. 2001; 276(48):45330–40. [PubMed: 11562363]
46. Scheen AJ. Clinical pharmacokinetics of metformin. *Clin Pharmacokinet*. 1996; 30(5):359–71. [PubMed: 8743335]
47. Zhou M, Xia L, Wang J. Metformin transport by a newly cloned proton-stimulated organic cation transporter (plasma membrane monoamine transporter) expressed in human intestine. *Drug Metab Dispos*. 2007; 35(10):1956–62. [PubMed: 17600084]
48. Christensen MM, Brasch-Andersen C, Green H, Nielsen F, Damkier P, Beck-Nielsen H, Brosen K. The pharmacogenetics of metformin and its impact on plasma metformin steady-state levels and glycosylated hemoglobin A1c. *Pharmacogenet Genomics*. 2011; 21(12):837–50. [PubMed: 21989078]
49. Higgins JW, Bedwell DW, Zamek-Gliszczynski MJ. Ablation of both organic cation transporter (OCT)1 and OCT2 alters metformin pharmacokinetics but has no effect on tissue drug exposure and pharmacodynamics. *Drug Metab Dispos*. 2012; 40(6):1170–7. [PubMed: 22407892]
50. Kirpichnikov D, McFarlane SI, Sowers JR. Metformin: an update. *Ann Intern Med*. 2002; 137(1): 25–33. [PubMed: 12093242]
51. Lin HZ, Yang SQ, Chuckaree C, Kuhajda F, Ronnet G, Diehl AM. Metformin reverses fatty liver disease in obese, leptin-deficient mice. *Nat Med*. 2000; 6(9):998–1003. [PubMed: 10973319]
52. Gangale MF, Miele L, Lanzone A, Sagnella F, Martinez D, Tropea A, Moro F, Morciano A, Ciardulli A, Palla C, Pompili M, Cefalo C, Grieco A, Apa R. Long-term metformin treatment is able to reduce the prevalence of metabolic syndrome and its hepatic involvement in young hyperinsulinaemic overweight patients with polycystic ovarian syndrome. *Clin Endocrinol (Oxf)*. 2011; 75(4):520–7. [PubMed: 21569072]
53. Palomba S, Falbo A, Carrillo L, Villani MT, Orio F, Russo T, Di Cello A, Cappiello F, Capasso S, Tolino A, Colao A, Mastrantonio P, La Sala GB, Zullo F, Cittadini E. Metformin reduces risk of ovarian hyperstimulation syndrome in patients with polycystic ovary syndrome during gonadotropin-stimulated in vitro fertilization cycles: a randomized, controlled trial. *Fertil Steril*. 2011; 96(6):1384–1390. [PubMed: 21982727]
54. Cheong JH, Park ES, Liang J, Dennison JB, Tsavachidou D, Nguyen-Charles C, Cheng KW, Hall H, Zhang D, Lu Y, Ravoori M, Kundra V, Ajani JA, Lee JS, Hong WK, Mills GB. Dual inhibition of Tumor Energy Pathway by 2-deoxy glucose and metformin Is Effective Against a Broad Spectrum of Preclinical Cancer Models. *Mol Cancer Ther*. 2011; 10(12):2350–62. [PubMed: 21992792]

55. Roe AL, Howard G, Blouin R, Snawder JE. Characterization of cytochrome P450 and glutathione S-transferase activity and expression in male and female ob/ob mice. *Int J Obes Relat Metab Disord.* 1999; 23(1):48–53. [PubMed: 10094576]
56. Sambol NC, Chiang J, O’Conner M, Liu CY, Lin ET, Goodman AM, Benet LZ, Karam JH. Pharmacokinetics and pharmacodynamics of metformin in healthy subjects and patients with noninsulin-dependent diabetes mellitus. *J Clin Pharmacol.* 1996; 36(11):1012–21. [PubMed: 8973990]
57. Hermann LS. Metformin: a review of its pharmacological properties and therapeutic use. *Diabetes Metab.* 1979; 5(3):233–45. [PubMed: 387488]

Figure 1. Decreased transporter activities of human OCT1 and MATE1 in the HEK-293 cells overexpressing IRIP

HEK-293 cells were cotransfected with a transporter expression plasmid and either an empty (empty-V) or IRIP (**A, B**: human IRIP; **C, D**: mouse IRIP) expression plasmid vector. MPP $^+$ (12 μM) uptake for 5 min was performed to assess the activities of OCT1 (**A, C**) and MATE1 (**B, D**), as described in the experimental section. The data represent mean $\pm$ SD from 3 replicates and were repeated thrice. * $P < 0.05$, ** $P < 0.001$ compared with control empty vector.

Figure 2. Decreased OCT1 protein expression by IRIP overexpression

HEK-293 cells were cotransfected with an OCT1-GFP plasmid vector and either an empty (empty-V, P5/FRT) or human IRIP plasmid vector. **A.** Fluorescence image of EGFP-OCT1 fusion protein. The pictures were taken 12 hours after the transfection with either 10 $\times$ or 20 $\times$ amplification. **B.** Cellular membrane expression of EGFP-OCT1. The membrane fraction was isolated from the HEK-293 cells. The primary antibody against GFP was used to detect the fusion protein (B. top panel). The immunoblots were quantified by densitometry with the density of the blank set as one unit. The results are averaged from three replicates (B: bottom panel). $**P < 0.001$ compared to Empty-V + EGFP-OCT1. **C.** Total cellular membrane proteins. The membrane fraction was isolated from the HEK-293 cells and loaded onto gel which was stained by Coomassie staining.

Figure 3. Increased transporter activity of human OCT1 in the HEK-293 cells with IRIP knockdown

A. The transcript levels of IRIP in the HEK-293 cells transfected with the plasmid containing human IRIP shRNA or control scramble shRNA. The results from two positive shRNA constructs (SH1 and SH8) and their combination (SH18) were shown; con, scramble shRNA; blk, blank transfection. **B.** MPP⁺ (12 nM) uptake in the HEK-293 cells transfected with the plasmids of human OCT1, along with empty vectors (empty-V), IRIP cDNA, scramble shRNA, or IRIP shRNAs. The data represent mean $\pm$ SD from 3 replicates and were repeated trice. * $P < 0.05$, ** $P < 0.01$ compared to controls.

Figure 4. Hepatic gene overexpression VIA hydrodynamic tail vein injection and effect of hepatic IRIP overexpression on metformin disposition in mice

A. GFP expression in the liver after hydrodynamic tail vein injection of the empty *pLive* and that containing GFP cDNA (*pLive-GFP*). **B.** The mRNA levels of mouse IRIP in the liver after the injection of the empty *pLive* plasmids and those containing mouse IRIP cDNA (*pLive-miRIP*). Quantitative RT-PCR was used to determine mRNA levels. * $P < 0.01$ as compared to *pLive*. **C.** Hepatic metformin accumulation in the mice injected with *pLive* and *pLive-miRIP*. The mice were euthanized 1 hour after metformin administration. **D.** Plasma metformin concentrations over time in the mice injected with *pLive* and *pLive-miRIP*. **B, C & D,** $n = 5-6$ mice/group. **C & D,** the mice were intraperitoneally administrated with metformin (15 mg/kg) 3 weeks after HTV injection.

Figure 5. Comparison of IRIP expression, metformin disposition, and Oct1 expression in obese *OB/OB* and lean littermates

A. Hepatic mouse IRIP transcript levels in *ob/ob* and lean mice. **B.** Hepatic membrane Oct1 protein levels in *ob/ob* and lean mice. Top panel: representative membrane Oct1 protein levels, detected by a mouse Oct1 antibody, in *ob/ob* (O1–4) and lean mice (L1–2). Bottom panel: the immunoblots were quantified by densitometry with the average density of the lean mice set as one unit. ** $P < 0.01$ compared to lean mice. **C.** Hepatic *Oct1* transcript levels in *ob/ob* and lean mice. **D.** Hepatic metformin accumulation in *ob/ob* and lean mice. 15 mg/kg metformin with ^{14}C tracer was given by i.p. injection. The mice were sacrificed 1 hour after metformin administration. **E.** Pharmacokinetics of metformin in *ob/ob* and lean mice. * $P < 0.05$, ** $P < 0.01$ compared to lean mice. A & C, quantitative RT-PCR was used to determine hepatic mIRIP mRNA levels. A - E, 5–7 mice per group were used.

Figure 6. Schema showing the effects of IRIP expression on the activities of basal uptake (e.g. OCTs) and apical efflux (e.g. MATEs) transporters, and their hypothetical consequences of drug (e.g. metformin) disposition

IRIP, ischemia/reperfusion-inducible protein; OCTs, organic cation transporters; MATEs, multidrug and toxin extrusion proteins; the chemical structure shown is metformin.

Table 1Pharmacokinetic parameters of metformin in lean mice (n = 6) and obese *ob/ob* mice (n = 6).^a

parameters	lean mice	<i>ob/ob</i> mice	<i>P</i> value ^b
AUC ₀₋₁₂₀ (μg/ml/min)	298 ± 47.3	333 ± 32.4	0.101
AUC _{0-∞} (μg/ml/min)	317 ± 46.2	400 ± 36.7	0.001
C _{max} (μg/ml)	7.06 ± 0.66	5.29 ± 1.17	0.001
T _{max} (min)	13.4 ± 3.09	22.5 ± 8.02	0.007
T _{1/2} (min)	20.2 ± 4.91	44.1 ± 6.83	< 0.001

^a 15 mg/kg metformin with ¹⁴C tracer was given by i.p. injection. The mice were sacrificed 2 hours after metformin administration.^b *P* < 0.05, significantly different from lean mice.