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PHARMACOGENOMICS OF MEMBRANE TRANSPORTERS: FOCUS ON THE ORGANIC CATION TRANSPORTER, OCT1

by

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To my wife, Jiayan,

for your unwavering belief in me and always cheering me up

To my parents,

who always believe that their son can make great accomplishments

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ABSTRACT

PHARMACOGENOMICS OF MEMBRANE TRANSPORTERS: FOCUS ON THE ORGANIC CATION TRANSPORTER, OCT1

Yan Shu

In mammalian cells, transmembrane flux of a variety of structurally diverse organic cations (e.g., metformin, tetraethylammonium, and 1-methy-4-phenylpyridinium) is mediated by organic cation transporters (OCTs). OCTs, including OCT1, OCT2 and OCT3, are thought to play a role in the absorption, distribution, elimination and tissue-specific targeting of organic cationic drugs. The goals of this dissertation were to characterize genetic variation in human *OCT1* (*SLC22A1*) and to determine the pharmacological and physiological roles of the transporter. The findings contribute to the broader goal of understanding the pharmacogenetics of membrane transporters.

The first part of this dissertation described the identification and functional characterization of genetic variants of *OCT1*. *OCT1* was found to be a highly polymorphic gene. Non-synonymous variants of OCT1 exhibited different rates of uptake of the model substrate MPP$^+$ in *Xenopus laevis* oocytes. The non-synonymous variants were also evaluated with respect to chemical change, evolutionary conservation, and amino acid substitution scoring matrices. The data suggest that genetic variation in *OCT1* may affect drug disposition.

The second part of this dissertation focused on determining the role of OCT1 in drug action. In particular, the role of OCT1 in the disposition and action of metformin, a drug used in the treatment of diabetes, was intensively investigated. Experiments in cell culture, *Oct1* knockout mice, and human volunteers demonstrated that OCT1 was required for the therapeutic effects of metformin. OCT1 was a determinant of response to metformin in cell lines and in mouse primary hepatocytes. The glucose-lowering effect of metformin was abolished in *Oct1*-deficient mice. Importantly, OCT1 function or genotypes has a significant impact on the pharmacokinetics and pharmacodynamics of metformin in animals and in healthy volunteers.

In the final part of this dissertation, the role of OCT1 in hepatic triglyceride homeostasis was explored. A series of assays and measurements were performed in obese (*ob/ob*) and lean (*OB/OB*) mice in the presence or absence of the *Oct1* allele. The data suggest that in addition to its function as a drug transporter, OCT1 is involved in nutrient, particularly triglyceride and glucose, homeostasis and may be important in the development of fatty liver.

Kathleen M. Giacomini

Jan 8, 2007

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CHAPTER 1

AN OVERVIEW OF ORGANIC CATION TRANSPORTERS

Overall Introduction to Transporters

Organisms have evolved complex mechanisms to maintain homeostasis of nutrients and to detoxify metabolic waste products and environmental toxins. These mechanisms include transport proteins, which selectively interact with their substrates and move them across membranes, and metabolic enzymes, which modify their substrates to facilitate their elimination. In humans, transport proteins have been increasingly recognized as important mediators for therapeutic agents to enter into and exit from the body. As such, many transport proteins are often termed drug transporters and are critical to the pharmacokinetics and pharmacodynamics of many therapeutic agents (1-3).

In general, transport proteins mediate the transfer of polar molecules across the hydrophobic membrane. While the majority (>80%) of transport proteins are oligospecific, accepting only nutritional and metabolic compounds with closely related structures, drug transporters are polyspecific, tending to interact with compounds of diverse sizes and structures (4). Several families of transport proteins have been extensively characterized as drug transporters. These include the ATP-binding-cassette (ABC) family represented by multiple drug resistance protein 1 (MDR1) (5), H^+-oligopeptide cotransporters of solute carrier family 15 (SLC15A1 and SLC15A2) (6), the solute carrier organic anion transporting polypeptide family (SLCO or SLC21A) (7), the concentrative nucleoside transporter family (SLC28A) (8), the equilibrative nucleoside transporter family (SLC29A) (9), and the organic cation-anion-zwitterion transporter family (SLC22A) (10, 11).

A variety of endogenous and exogenous compounds can be classified as organic cations, anions and zwitterions. The SLC22A protein family is dedicated to the translocation of these polar hydrophilic molecules across the plasma membrane (10, 11). In humans and rodents, the SLC22A family can be divided into distinct subfamilies or subgroups according to sequence homology. Phylogenetic tree analysis reveals that the SLC22A family has at least three subfamilies: organic cation transporters (OCTs), organic anion transporters (OATs), and novel organic cation transporters (OCTNs) that are capable of transferring carnitine and/or organic cations. Most transporters in the SLC family are polyspecific and regarded as drug/xenobiotic transporters. This dissertation focuses on the OCT subfamily. In particular, the pharmacogenetics and physiology of OCT1, a member of the OCT subfamily, have been intensively investigated.

Currently, the OCT subfamily has three members: OCT1, OCT2 and OCT3. These three transport proteins share similar molecular characteristics, transport mechanisms and substrate specificities. However, they are also distinct from each other with respect to these characteristics and others such as regulation, physiology, and genetic polymorphisms. It is noteworthy that whereas OCT3 is mainly documented as an extraneuronal transporter for neurotransmitters (12, 13), OCT1 and OCT2 can interact with a variety of structurally diverse organic cationic compounds (4, 10, 14, 15). These compounds include clinically used drugs (e.g., metformin), endogenous compounds (e.g., dopamine), as well as toxic substances (e.g., 1-methy-4-phenylpyridinium, MPP$^+$). Some substrates for OCT1 and OCT2 are clinically important drugs. For example, metformin is a first-line therapy for type 2 diabetes (16). Therefore, OCT1, OCT2 and even OCT3 are clinically important as they may play a role in pharmacokinetics and pharmacodynamics, and therefore, in determining therapeutic effects for certain clinically used drugs. In this dissertation, metformin is another focus, and the pharmacogenetic significance of OCT1 is explored by studying this widely prescribed drug.

This introductory chapter is divided into two major sections: **I.** Introduction to the organic cation transporters; and **II.** Research rationales and summary of chapters in this dissertation. In the first section, following a brief introduction to mammalian organic cation transport systems, the molecular cloning of the OCTs, their tissue distribution, transporter mechanisms, substrate specificities and regulation are reviewed. The gene knock-out mouse models and the genetic polymorphisms of human *OCTs* are also presented. Finally, future directions for clinical studies on this class of transporters are discussed. The second section presents research rationales, specific background and research summary for this dissertation. Following an introduction to the overall rationale of performing pharmacogenetic studies on drug transporters, the pharmacokinetics and pharmacodynamics of metformin are reviewed. In addition, the current understanding of molecular mechanisms of metformin action is introduced. At the end of this chapter, a summary of the work in each research chapter in this dissertation is presented.

I. Introduction to Organic Cation Transporters

General background. The organic cation transport systems were initially recognized in studies of renal elimination. A variety of endogenous and exogenous compounds that are harmful to the body are eliminated from the kidney *via* glomerular filtration and/or active tubular secretion. Among those compounds are polar organic cations that require a transporter-mediated process to cross cellular membrane and be eventually excreted into the urine. The organic cation transporter systems in the kidney have been recognized for several decades (14, 17, 18). Prior to the molecular cloning of various renal transporters, it had been demonstrated that there are unidirectional transcellular transport systems responsible for secreting low molecular weight organic cations, primarily through proximal tubule cells and, to a less extent, through distal tubule cells. The systems involve the uptake of organic cations into the cells from the blood across the basolateral membrane, which is mediated by several polyspecific, membrane potential-sensitive transport mechanisms, and the efflux of organic cations into the tubular lumen fluid across the brush-border membrane, which is *via* H^+- or cation-exchange antiport mechanisms. Following the insight gained from the kidney, organic cation transporters have also been characterized in other tissues (15, 19). In particular, the organic cation transporters in the liver and in the intestine have been proposed as important determinants of drug absorption and disposition. Over the past several decades, the mechanisms of organic cation transport have been extensively characterized using *in vivo* models, *ex vivo* organ perfusions, *in vitro* tissue preparations and cell lines. In particular, the molecular cloning and characterization of organic cation transporters have remarkably increased our knowledge of this important subfamily of solute carriers. Below, the recent progress in understanding various aspects of the organic cation transporters (OCTs) is described, beginning with the cloning of the first OCT in 1994.

Molecular cloning and characterization. In 1994, the first organic cation transporter, rOCT1, was cloned by the Grundemann group from rats (20). rOCT1 was also the first cloned member of the current SLC22A family. Soon after the cloning of rOCT1, other OCT isoforms in mammals were cloned using homology cloning strategies (11). Currently, there are three cloned members in the OCT subfamily: OCT1, OCT2 and OCT3. The orthologs of mouse, rat, rabbit, pig, dog, and human have been cloned for one or all of the three OCTs (21). The OCTs are usually 500-600 amino acids in length. There is more than 80% sequence identity between human and rodent orthologs for each of the OCTs. The identity is usually more than 50% between different OCT paralogs within a species.

Like most transporters in the SLC22A family, the predicted secondary structure of the OCTs, based on sequence analysis and hydropathy profile, has 12 α-helical transmembrane domains (TMDs), a large glycosylated extracellular loop between the first and second TMD, and a large intracellular loop between the sixth and seventh TMD. This is represented in Figure 1.1 by the predicted human OCT1 topology. There are multiple potential glycosylation and phosphorylation sites within the two loops. These sites have been associated with the regulation of transport function by other proteins (22). rOCT1/rOAT3 mutagenesis studies from this laboratory suggest that the residues involved in substrate recognition reside within TMDs 6-12, the carboxyl-terminal half of OCTs (23, 24). In contrast, recent evidence suggests that the fourth and tenth TMDs are critical to substrate recognition for the OCTs (25-27). Because OCTs are polyspecific transporters and have broad substrate selectivity, it is expected that different substrates

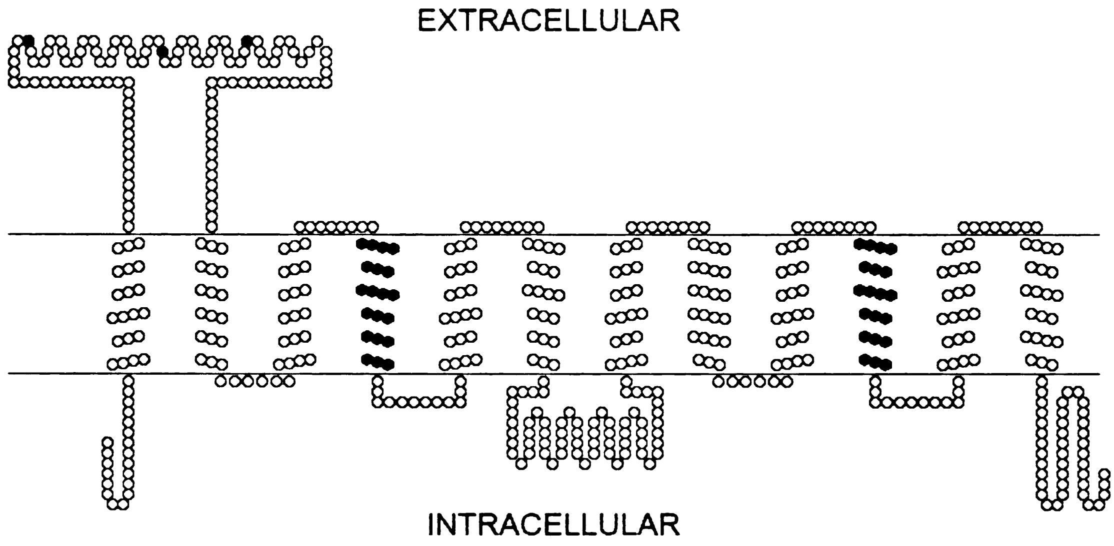

Figure 1.1. Predicted secondary structure of OCTs. Shown here is the transmembrane topology of human OCT1. The secondary structure was rendered using the software of TOPO. Human OCT1 shares a similar transmembrane topology with the other OCT orthologs and homologs. Human OCT1 has 12 α-helical transmembrane domains (TMDs), a large glycosylated extracellular loop between the first and second TMD, and a large intracellular loop between the sixth and seventh TMD. The putative N-glycosylation sites in the extracellular loop are represented by dark circles. TMD4 and TMD10 (dark circles) were suggested to be critical to substrate recognition (25-27).

may be recognized by different sites and that multiple substrate recognition sites may exist.

The molecular cloning of OCTs and the sequencing of the human genome have facilitated the genomic localization of OCTs. The human *OCT* genes are localized to chromosome 6q, the mouse to chromosome 17, and the rat to chromosome 1q (11). *OCT1*, *OCT2* and *OCT3* are clustered on the chromosome, and each gene usually consists of 11 exons and 10 introns.

Transport mechanisms. Studies on the transport mechanism of OCTs have revealed that OCTs are usually facilitators driven by the inside-negative membrane potential (28, 29). OCTs are not coupled to a free energy source. The transport of organic cations by OCTs is saturable, dependent on the electrochemical gradient across the membrane, and sensitive to membrane potential, as demonstrated in the transport studies of altering membrane potentials. For example, the transport rates can be remarkably reduced by the treatment with ionophores such as valinomycin that abolish the membrane potential. Electrophysiological studies in this laboratory and others directly demonstrated that the uptake of organic cations by OCTs causes inward currents (30-32). OCTs are bidirectional transporters *per se*. Although the physiological membrane potential favors the entry of positively charged substrates into the cell, the direction of substrate flux is actually determined by the overall electrochemical gradient across the membrane. As such, OCTs may serve as efflux transporters, given a higher in to out electrochemical potential energy across the cell membrane. In addition, OCTs can act as electroneutral organic cation-cation exchangers; that is, outwardly directed gradients of organic cations can drive the inward movement of organic cations and *vice versa*. (30, 31, 33). OCT activity is insensitive to sodium and pH gradients *per se*. However, because pH can affect the ionization of organic cations, transport rates of weak bases may apparently depend on pH.

Tissue distribution and localization. Differences in tissue distribution among OCTs may be a major cause of physiological and pharmacological differences in this subfamily of transporters. In humans, OCT1 is predominantly expressed in the liver, but also in the heart, skeletal muscle, kidney, placenta and small intestine (34, 35). Human OCT2 is mainly expressed in the kidney, with lower expression in other tissues such as placenta, thymus, adrenal gland, neurons and choroid plexus (33, 34). In general, OCT1 is described as a "liver-specific" organic cation transporter, while OCT2 as "kidney-specific". In contrast, human OCT3 has a broad expression pattern (36). Originally characterized as a transporter for multiple neurotransmitters such as dopamine, epinephrine, and norepinephrine in non-neuronal tissues, OCT3 is thought to be the "extraneuronal monoamine transporter" (EMT) (13). However, the name may be misleading because OCT3 has been found to be expressed in neurons as well (36). Recent microarray data confirmed the early expression data of OCTs by Northern blotting and RT-PCR. In particular, quantitative comparisons indicate that human OCT1 and OCT2 have an extremely high expression in the liver and kidney, respectively, with background or near background levels in all other tissues (37). However, despite overall similar expression patterns across mammalian OCTs, there are species differences in tissue specificities. For example, in rodents, "liver-specific" Oct1 has a significant level of expression in the kidney that is comparable to that of the "kidney-specific" Oct2 (20).

OCTs have been thought to localize to the basolateral membranes of polarized cells including epithelia from the liver and kidney. In the liver, OCT1 was found to be expressed in the sinusoidal (basolateral) membrane by immunohistochemistry and Western blot (38), consistent with its function as a sinusoidal uptake transporter taking cationic drugs from the blood (Figure 1.2). As discussed above in the transport mechanism, bidirectional OCT1 may also participate in the release of organic cations across the sinusoidal membranes into the blood. This may be significant as the liver is a major detoxification organ where drug-metabolizing enzymes catalyze the biotransformation of hydrophobic compounds to hydrophilic metabolites, including organic cations. Excretion of organic cations across the biliary (apical) membrane into the bile is suggested to be mediated by a H^+-organic cation antiporter system (39) and MDR1 (5). The recently cloned multidrug and toxin exclusion transporter 1 (MATE1) is a H^+-organic cation exchanger located at the biliary membrane (40, 41).

In humans, OCT2 has been localized to the basolateral membrane of proximal tubules in the kidney (42) (Figure 1.3). In rodents, both Oct1 and Oct2 were found at the basolateral membrane in renal proximal tubules (43-45). The localization is consistent with the notion that OCT2 and, in rodents, Oct1 as well, mediate the first step of renal active secretion of organic cations. These transporters take the organic cations from the blood across the basolateral membrane into the tubule cells. Similar to those in the liver, there are efflux transport systems in the apical membrane of tubule cells responsible for excretion of organic cations from the tubular cells into the tubular lumen. Several

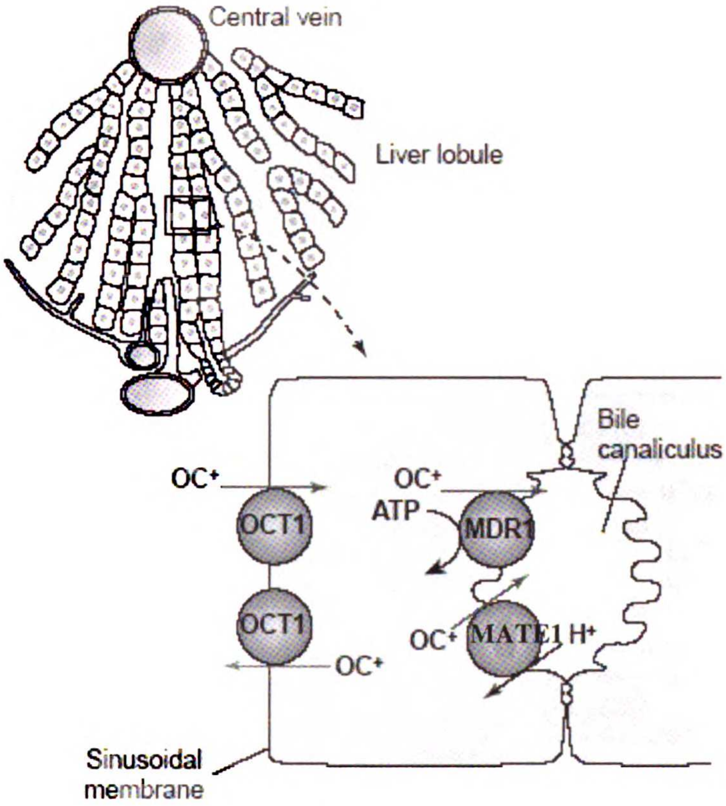

Figure 1.2. Model of organic cation transport in the liver. OCT1 is by far the most abundant organic cation transporter in the liver, where it serves as a sinusoidal (basolateral) uptake transporter. After entry into the hepatocyte, organic cations may be metabolized or excreted into bile *via* the efflux transporters such as the multidrug resistance protein 1 (MDR1) and the multidrug and toxin exclusion transporter 1 (MATE1), which is an organic cation-H^+ exchanger, in the biliary (apical) membrane. OCT1 may also mediate the translocation of organic cations out of hepatocytes into sinusoids. The figure was adapted from that by Koepsell (4).

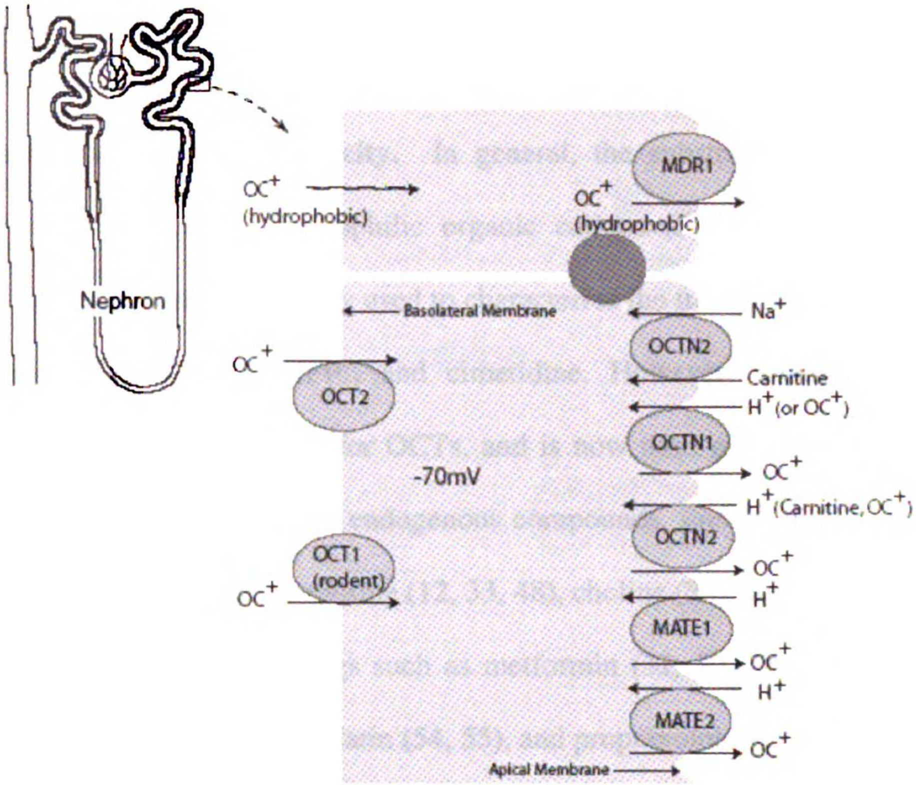

Figure 1.3. Model of organic cation transport in the kidney. Organic cations are filtered in the glomeruli and/or secreted in the proximal tubule, and to a less extent, in the distal tubule. Filtered organic cations could be reabsorbed in the apical membrane of tubule cells. In humans, OCT2 is the primary transporter responsible for uptake of organic cations across the basolateral membrane. In rodents, both Oct1 and Oct2 are the basolateral uptake transporters. Efflux of organic cations across the apical (luminal) membrane for excretion is mediated *via* several transporters including the cation proton exchanger OCTN1, the Na$^+$-carnitine cotransporter (OCTN2), the multidrug and toxin exclusion transporter 1 (MATE1) and 2 (MATE2), and the multidrug resistance protein 1 (MDR1). The figure was adapted from that by Koepsell (4) and that by Thomas J. Urban in our laboratory.

transport proteins including MDR1 (46), OCTN1 (10), OCTN2 (10), MATE1 (40) and MATE2 (41) have been implicated in the efflux process (Figure 1.3).

Substrate/inhibitor specificity. In general, the substrates of OCTs are low molecular weight, relatively hydrophilic organic cations (Figure 1.4). The common substrates that have been extensively used to characterize the transporter function include tetraethylammonium (TEA), MPP^+, and cimetidine. However, cimetidine has been reported as only a weak substrate for OCTs, and is now used primarily as an inhibitor (47). Other OCT substrates include endogenous compounds, such as dopamine (33, 48), epinephrine (12, 33, 48), norepinephrine (12, 33, 48), choline (34), and histamine (33, 49), and exogenous clinically used drugs such as metformin (32, 50, 51), phenformin (50), pindolol (52), rantidine (53), oxaliplatin (54, 55), and propranolol (56). It should be noted that while most OCT substrates are organic cations and weak bases, some are organic anions or neutral compounds at physiological pH. For example, anionic prostaglandin E_2 and prostaglandin $F_{2\alpha}$ are transported by both OCT1 and OCT2 (57). In addition, because of the difficulty in measuring transport *per se* (e.g., a radioactive isoform is usually required to determine transportability of a compound), many more inhibitors than substrates of OCTs have been characterized. Typical OCT inhibitors include quinidine (58), quinine (34, 58), desipramine (20, 34, 58), clonidine (13, 58), and procainamide (20, 58). The substrates and inhibitors of OCTs are reviewed in detail by others (10, 14).

There is extensive overlap among OCTs with respect to the substrate and inhibitor specificities. However, distinct differences in affinity and maximal transport rate for substrates and/or inhibitors exist between OCT paralogs and species orthologs. For

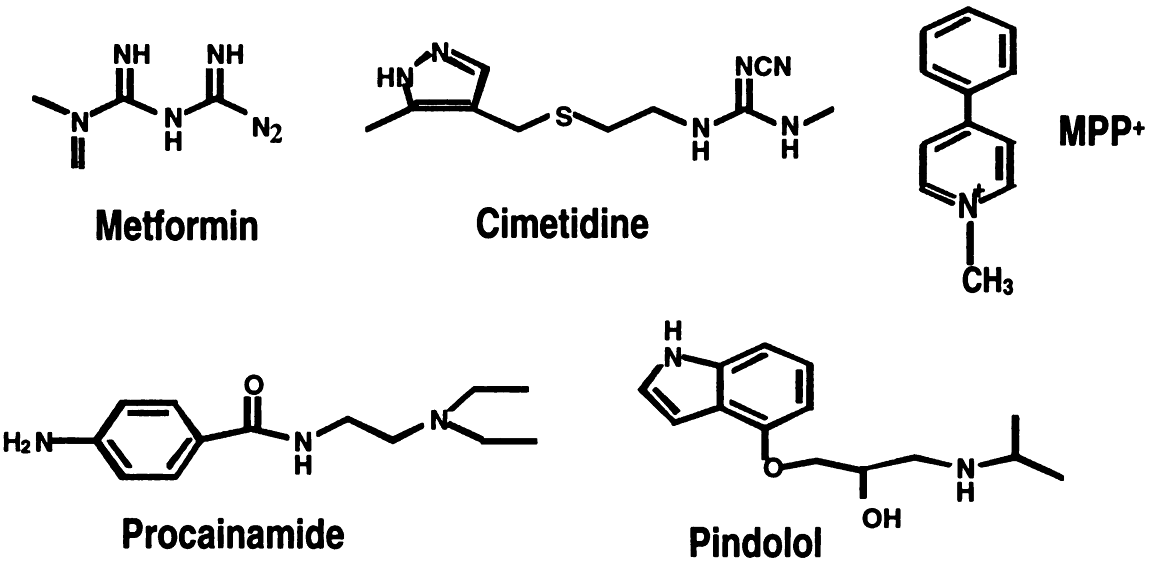

Figure 1.4. Structurally diverse organic cations interact with OCTs. OCTs are capable of transporting a variety of structurally diverse substrates, including endogenous compounds, environmental toxins and clinically important drugs.

example, while both human OCT1 and OCT2 transport the anti-diabetic drug metformin, the drug is not a substrate for human OCT3 (Figure 1.5, unpublished data from this laboratory). Even between OCT1 and OCT2, metformin is a better substrate for OCT2 with a higher affinity (59). Other examples of substrate/inhibitor specificity among OCT paralogs include corticosterone, which has an IC_{50} (concentration for half-maximal inhibition) of 10, 30, and 0.1 μM for human OCT1, OCT2 and OCT3, respectively (10), procaninamide, which shows a much higher affinity for rat OCT1 compared to rat OCT2 (20, 60), and even TEA, which is a much weaker substrate for OCT3 than for the other two OCT isoforms (10, 12, 13). While it can efficiently transport some endogenous biogenic amine neurotransmitters such as dopamine, epinephrine, norepinephrine, histamine, and serotonin, OCT3 appears to be a poor transporter for xenobiotics. This is consistent with the tissue distribution patterns of OCTs. OCT1 and OCT2 are mainly expressed in the liver and kidney, both of which are important organs for detoxification of endogenous metabolites and xenobiotics. OCT3 is broadly expressed and thought to be involved in extensive clearance of endogenous catecholamines in extraneuronal tissues (13).

OCT orthologs from different species may also exhibit differences in substrate/inhibitor specificity. A good example comes from this laboratory in studies of characterizing the interaction of a series of n-tetraalkylammonium (nTAA) compounds with OCT1 orthologs of mouse, rat, rabbit and human (31). The nTAA compounds have different lengths of alkyl chains and thus different molecular weights. In general, human OCT1 has a weaker affinity to nTAA compounds compared to other orthologs. However, larger nTAA compounds are transported more efficiently by human OCT1, whereas

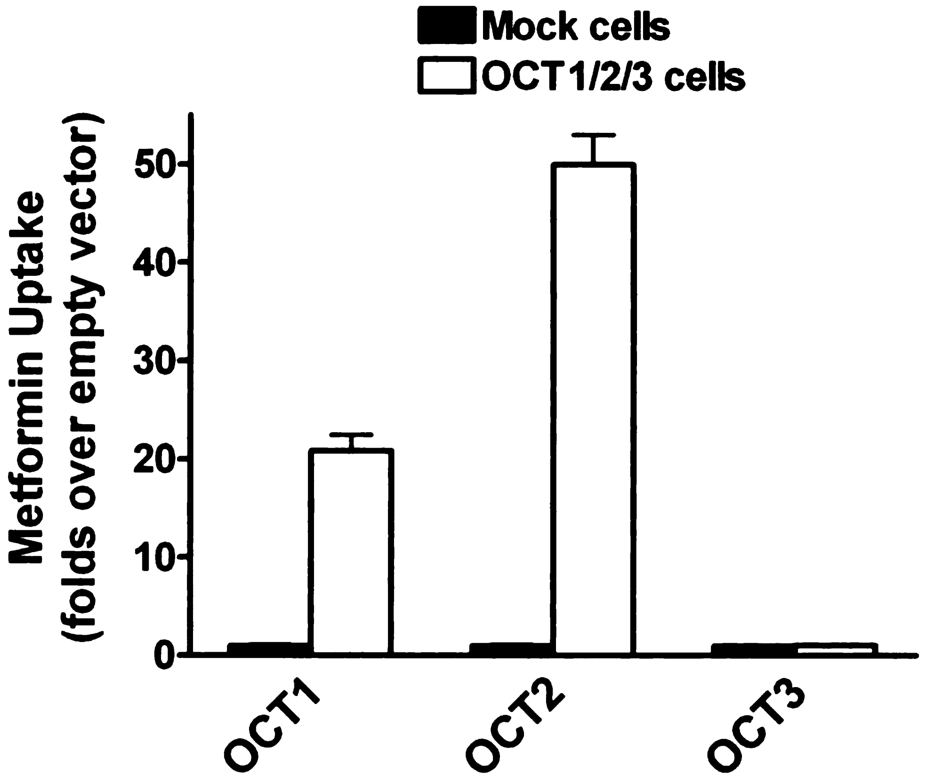

Figure 1.5. OCTs may exhibit different transport rates for the same substrates, as exemplified by metformin. Human OCT1, OCT2 and OCT3 were stably over-expressed in HEK-293 cells. Mock cells were those transfected with an empty vector. Metformin (250 μM) uptake was measured at 10 minutes. The unpublished data are consistent with the finding from Kimura *et al.* that metformin is a superior substrate for human OCT2 rather than OCT1 (59).

smaller nTAAs are transported at greater rates by mouse OCT1 or rat OCT1. The rabbit OCT1 exhibits intermediate properties in its interactions with nTAAs compared with the rodent and human orthologs. The species differences in substrate specificity underscore potential difficulties in extrapolating data from animals to humans.

Regulation of OCTs. Although the pharmacological roles of OCTs have been extensively studied, their physiological functions remain to be characterized. Understanding their physiological function requires knowledge of their regulation. The regulation of OCT expression and activity occurs at different levels, including post-transcriptional modification at protein levels, transcriptional modulation by transcriptional factors and hormones, dynamic expression change during development and expression alteration by disease status (Table 1.1).

Currently, the most abundant evidence for OCT regulation is obtained at the protein level in studies examining the effects of protein kinases and/or phosphatases using cell culture models, isolated proximal tubules and cell culture systems stably expressing different OCT isoforms (22). There are multiple potential phosphorylation sites that are conserved among OCTs. These phosphorylation sites provide target sequences for functional regulation by kinases and phosphatases. It has been demonstrated that activation of protein kinase A (PKA) increases rat OCT1 transport activity (61) and reduces the transport activities of human OCT1 and OCT2 (62, 63) but not OCT3 (64). Activation of protein kinase C (PKC) also enhances rat OCT1 transport activity (61) but does not influence the human OCT activities (62-65). In addition, activation of protein kinase G (PKG) decreases the transport activities of rat OCT1 and

Table 1.1. Summary of the regulation of OCTs by different factors.

Regulation factors	Mouse			Rat			Human			Ref.
OCT isoforms	1	2	3	1	2	3	1	2	3	
Post-transcriptional Modification										
PKA				+			-	-	+/-	61-64
PKC				+			+/-	+/-	+/-	61-64
PKG				-			+/-	-	+/-	63, 65
Tyrosine kinase				+			+			61, 63
CaM							+	+	+	63-65
CamMII							+	+	+	62-64
MLCK							+/-	+		62, 63
PI3K							+/-	-		62, 63
PDE1									+	64
Tyrosine phosphatase									+	64
ALP									+	64
Serine/theronine phosphatase									+	64
Transcriptional Factors/Hormone										
*HNF1α -/-	+, -	+	+							69
HNF4α							+			70
SHP							-			70
PPAR α	+									71
PPARγ	+									71
Testosterone					+					72-75
Development and Diseases										
Embryonic development	+			+						76-78
Chronic renal failure					-					79
Diabetes				-	-	-				80
Hyperuricaemia					-					81
Obstructive cholestasis	-									82
Behavioural sensitization						-				83
Pre-eclampsia									-	84
Serotonin transporter -/-	+		+							85, 86

+, upregulation; -, down-regulation; +/-, no effect.

*Mouse OCT1 in the liver is upregulated, while that in the kidney downregulated.

Abbreviations: PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; CaM, calmodulin; CaMII, CaM-dependent kinase II; MLCK, myosin light-chain kinase; PI3K, phosphoinositide 3-kinase; PDE1, phosphodiesterase-1; ALP, alkaline phosphatase; HNF1α, hepatocyte nuclear factor 1α; HNF4α, hepatocyte nuclear factor 4α; SHP, small heterodimer partner; PPAR-α and -γ peroxisome proliferator activated receptor-α and –γ.

human OCT2, with no effect on human OCT1 and OCT3 (66). It seems that OCT activity is related to intracellular concentrations of Ca^{2+}, which is an important second messenger. Carbachol, a muscarinic acetylcholine receptor agonist that increases the intracellular concentration of Ca^{2+}, can inhibit human OCT2 activity (62). The underlying mechanism is to activate the phospholipase C (PLC) pathway *via* phosphatidylinositol 3-kinase. In addition, the Ca^{2+}–calmodulin complex can activate human OCT1, OCT2 and OCT3 (62, 67). Human OCT3 is active in the dephosphorylated state that is regulated by tyrosine phosphatase, protein serine/threonine phosphatase, alkaline phosphatase, or the Ca^{2+}–calmodulin pathway (64). There are also potential glycosylation sites in the OCTs. Glycosylation at these sites may affect transport activity and provides additional mechanisms for OCT regulation, as evidenced by mutation analysis of three rabbit OCT2 glycosylation sites (68).

The expression of OCTs can be regulated at the transcriptional level by transcription factors and hormones. Disruption of the transcription factor hepatocyte nuclear factor 1α (HNF1α) in mouse altered the expression levels of many transporters (69). As for OCTs, the expression of both OCT1 and OCT3 in the liver and OCT2 in the kidney was increased, but OCT1 expression in the kidney was reduced (69). It has been reported that the transcription of human *OCT1* is activated by HNF4α and suppressed by bile acids *via* the bile acid-inducible transcriptional repressor, small heterodimer partner (SHP) (70). In addition, the agonists of peroxisome proliferator activated receptor (PPAR)-α and -γ transcriptionally increase *OCT1* expression in mouse and in cell lines (71). OCT regulation by hormones is evidenced by the fact that renal expression of rat OCT2 is gender dependent (72) and altered with administration of steroids (73). Steroids also alter the expression of dog OCT2 in Madin-Darby canine kidney (MDCK) cells (74). Recently, Asaka *et al.* reported that the induction of rat OCT1 by testosterone is *via* the androgen receptor-mediated transcriptional pathway (75).

Regulation of OCT expression is also modulated during development and in certain disease states. In mice, high levels of OCT1 transcripts are at first detected in the ascending aorta and heart atrium between embryonic (E) days E14 and E16 (76). After E16, the kidney and then the liver gradually become the major organs of OCT1 expression (76). In rats, the level of transcripts of OCT1 along with two other SLC22A family members, OCTN1 and OCTN2, increase gradually from postnatal (P) day P0 to P45 (77). Rat OCT1 expression may be a potential marker of terminal differentiation for proximal tubulogenesis in the kidney (78). OCT expression can be altered by disease states. Evidence so far indicates down-regulation of one, two or all OCTs in most disease states studied including chronic renal failure (79), diabetes (80), hyperuricaemia (81), obstructive cholestasis (82), behavioural sensitization (83) and pre-eclampsia (84). However, it has been reported that there was tissue-specific up-regulation of OCT1 and OCT3 in the serotonin transporter-deficient mice (85, 86).

Knockout mouse models. Giving their interaction with a variety of structurally diverse compounds, including clinically used drugs and their tissue distribution in the liver, kidney and intestine, OCTs have been proposed as critical determinants for the pharmacokinetics and pharmacodynamics of certain compounds. However, direct evidence from *in vivo* studies is required to support this idea. In addition, although OCTs have been cloned for a decade, their physiological functions are still poorly understood.

With the hope of a better understanding of OCT function, knockout mouse models have been generated for all of the three cloned OCTs (OCT1-3) (87-89).

OCT knockout mice (*Oct1-/-*, *Oct2-/-*, *Oct1/2-/-* and *Oct3-/-* mice) are viable and fertile, showing no obvious physiological defects (87-89). However, there were significant differences in the disposition of organic cations in these mice as compared to the wild-type control mice. When administered intravenously, TEA, MPP^+, metaiodobenzylguanidine (MIBG) and metformin accumulate much less in the liver and small intestine of *Oct1-/-* mice (50, 88). The biliary intestinal excretion of TEA is reduced in *Oct1-/-* mice, whereas the renal excretion is increased (88). In *Oct2-/-* mice, the accumulation of intravenous TEA in tissues such as liver, small intestine, and kidney is similar to wild-type mice, with the exception of a borderline decrease in the brain for *Oct2-/-* mice (89). There is no difference in the renal excretion of TEA between *Oct2-/-* and wild-type mice. This may be explained by the significant OCT1 expression in rodent kidney and compelled the researchers to generate *Oct1/2-/-* double knockout mice (89). In *Oct1/2-/-* mice, the active tubular secretion of TEA is abolished, resulting in approximately 6-fold higher steady-state plasma TEA concentrations as compared to *Oct1-/-*, *Oct2-/-*, or wild-type mice infused intravenously with TEA (89). The data indicate that OCT1 is crucial for hepatic and intestinal uptake of certain organic cations, whereas OCT1 and OCT2 together are necessary for renal clearance of these compounds in rodents. In *Oct3-/-* mice, the tissue distribution of monoamine MPP^+ was reduced by approximate 75% in the heart (87). In addition, after intravenous injection of MPP^+ in *Oct3+/-* heterozygous females impregnated in a heterozygous cross, the accumulation of MPP^+ in *Oct3-/-* embryos was about 3-fold lower compared with wild-type embryos (87).

These data suggest that OCT3 acts as an uptake transporter for monoamines or certain organic cations in the heart and at the fetoplacental interface.

It seems that OCTs are not essential for rodent normal physiology as evidenced by the viable, fertile and healthy *Oct*-knockout mice. It is noteworthy, however, that many subtle physiologic phenotypes or aberrations only become amplified by specific or extreme conditions. This appears to be the case for OCT3. Vialou *et al.* reported that *Oct3-/-* mice had an increase in the level of ingestion of hypertonic saline under thirst and salt appetite conditions, and the neural response to sodium deprivation in the subfornical organ of *Oct3-/-* brain was different from that of wild-type mouse brain (90). These data indicate that OCT3 is critical for the balanced neural and behavioral responses to environmentally induced variations in osmolarity. It was reported recently that OCT3 was a histamine transporter and participated in the control of basophil functions in *Oct3-/-* mice (91). The data imply that OCT3 may be involved in allergic diseases. Moreover, after treated with lipopolysaccharide (LPS) to induce endotoxemia, the survival rate of *Oct3-/-* mice was significantly lower compared to wild-type mice (92). This was explained by a higher immunological reaction *via* the increased histamine content in the spleen of *Oct3-/-* mice. In addition, by using *Oct*-knockout mouse models, Kummer *et al.* demonstrated that OCT1 and/or OCT2 mediate acetylcholine release from the respiratory epithelium, whereas OCT3 is involved in the reduction of 5-HT-induced bronchoconstriction by corticosterone (93). However, the primary physiological roles of OCT1 and OCT2 remain to be determined as they are predominantly expressed in the liver and kidney. In chapter 5 of this dissertation, we explored the role of OCT1 in triglyceride and glucose homeostasis using *Oct1-/-* mice.

Genetic variation of human OCTs. With the increasing recognition of drug transporters as pharmacokinetic and pharmacodynamic determinants, the genetic variants in drug transporter genes have been extensively studied as a molecular basis for interindividual variability in response to drugs. Recent wide-scale sequencing analysis and high-throughput genotype methods have dramatically accelerated the detection of genetic variants. For the past five years, numerous genetic variants have been reported in human *OCTs*, followed by functional characterization of the variants in heterologous expression systems.

For *OCT1*, in a population of 57 healthy Caucasians, Kerb *et al.* identified 25 genetic variants, eight of which resulted in a change in protein sequence (94). By measurement of transport activity in RNA-injected *X. laevis* oocytes, they found three variants of reduced transport activity (Arg61Cys, Cys88Arg, and Gly401Ser). As part of this dissertation research, we identified 15 nonsynoymous variants, along with numerous synonymous and intronic variants, in 247 DNA samples from ethnically diverse healthy subjects (Chapter 2) (95). By performing uptake of MPP^+ in *X. laevis* oocytes expressing the nonsynonymous variants, Arg61Cys, Pro341Leu, Gly220Val, Gly401Ser and Gly465Arg were found to exhibit significantly reduced transport function, while Ser14Phe show increased transport. Interestingly, Ser14 corresponds to the likely ancestral allele, as phenylalanine was found at this position in all available mammalian OCTs. Recently, Ser14Phe and another variant, 420del, were found to have reduced transport of metformin in HEK-293 stable cells, while Pro341Leu had normal activity for metformin (see Chapter 3, unpublished data), suggesting substrate specificity for different variants. Additional genetic variants including two having reduced transport function (Pro283Leu and Arg287Gly) are identified in Japanese individuals (96, 97). Human *OCT1* is thus a highly variable gene in terms of genetic polymorphism and function of the polymorphisms. It should be noted that some of the reduced function variants are common in populations. For example, 420del has an allele frequency of 18.5% in American Caucasians (95). These common functional variants provide attractive tools for studying pharmacogenetics of OCT1, as demonstrated in Chapters 3 and 4 of this dissertation.

Genetic variants of human *OCT2* have also been identified and functionally characterized in this laboratory (98). Of the 28 variants identified in 247 DNA samples, eight caused amino acid substitutions and one was a single-nucleotide insertion resulting in a premature stop codon. The transport function of the model organic cation, MPP^+, was evaluated in *X. laevis* oocytes expressing the eight nonsynonymous variants and the truncated OCT2. The truncated OCT2 has no transport function. While Lys432Gln displayed a higher affinity for MPP^+, Met165Ile and Arg400Cys exhibited lower maximal transport capacity (V_{max}), as compared with the reference OCT2. Ala270Ser, a common variant with an allele frequency of 12.7% among the ethnically diverse groups, showed increased affinity to the OCT inhibitor tetrabutylammonium (TBA), while Arg400Cys and Lys432Gln had decreased affinity. Additional single nucleotide polymorphisms (SNPs) in the *OCT2* gene were identified in 48 unrelated Japanese individuals (99) and 116 arrhythmic Japanese patients (100). The effects of these SNPs on transporter function remain to be determined.

Genetic variation in *OCT3* has been reported by Lazar *et al.* in 100 healthy individuals of European descent (101). Six single-nucleotide substitutions and one deletion were discovered in the core promoter, the exonic and flanking intronic sequences and the 3'-untranslated region. No amino acid changes were found. The functional consequences of these variants are unknown. It is suggested that the synonymous 1233G>A substitution may cause a cryptic 3'-splice acceptor site (101). Research from the Pharmacogenetics of Membrane Transporters Investigators identified 14 SNPs in 247 DNA samples (102). Of these, three resulted in amino acid substitutions (102), but no functional change was detected using a *X. laevis* oocytes expression system and MPP^+ as the model substrate (unpublished data). Compared to *OCT1* and *OCT2*, *OCT3* is less variable and seems to be more evolutionarily conserved.

Conclusions and clinical perspectives. The organic cation transport systems have been well studied for several decades. In particular, great progress has been made since the ascertainment of molecular identities for OCTs. Three OCTs (OCT1, OCT2, and OCT3) have been cloned and these facilitated transporters are driven by the electrochemical gradient or cation-cation exchange to transport a variety of cationic compounds. The tissue distribution and cellular localization of these transporters are consistent with their recognized physiological and pharmacological roles: excretion of xenobiotics and clearance of endogenous cationic metabolites. OCT1, a primary sinusoidal uptake transporter, may be important for the distribution of drugs into the liver to access metabolizing enzymes and for excretion into bile. OCT1 may also mediate the release of hydrophilic cationic metabolites from hepatocytes to blood. Kidney-specific

OCT2 is one of the primary mechanisms responsible for the basolateral uptake of organic cations into renal tubules for active secretion. Broadly expressed OCT3 seems not to be an efficient xenobiotic transporter but a transporter dedicated to extraneuronal clearance of catecholamines. The studies with the *Oct*-knockout mice have clearly demonstrated that for certain substrate drugs, the OCTs play an important role in pharmacokinetic processes such as hepatic distribution and biliary excretion (OCT1), renal secretion (OCT1 and OCT2), and heart accumulation and transport across placenta (OCT3). The implication of OCTs in drug disposition has led to studies identifying and characterizing genetic variation in human *OCT* genes. Numerous genetic variants with a transport function change have been discovered for human *OCT1* and *OCT2*. It is of great interest to examine drug disposition and response in the individuals who carry these variants in clinical studies exemplified by those presented in the chapters three and four of this dissertation.

Because OCTs are critical in the biodistribution of cationic drugs, it is straightforward that factors affecting OCT function are determinants of pharmacokinetics and pharmacodynamics. Genetic variation in *OCT1* and *OCT2* has been demonstrated to cause transport function change and thus might be an important factor. Moreover, a great number of drugs have been characterized as OCT substrates and/or inhibitors. Co-medication with these drugs may cause OCT-based drug-drug interactions. In addition, the regulation of activity and expression level of OCTs through protein kinase activators, PPARα agonists, gender, and disease states may affect the pharmacokinetics and pharmacodynamics of substrate drugs.

The physiological roles of OCTs are still unclear. *Oct*-knockout mice show no physiological defects. It is proposed that the physiological aberrations by Oct function deficiency may be subtle and become only apparent under specific conditions. As reviewed above, some interesting phenotypes in *Oct3-/-* mice have been revealed under particular circumstances. Therefore, further studies of the *Oct*-knockout mice under different circumstances are needed to fully understand OCT pathphysiology. Since numerous genetic variants, including common ones, have been identified in the human *OCT* genes, it will also be of great interest to perform genetic studies in special populations such as those under a particular disease state. Such studies may be useful to determine the role of OCTs in pathophysiology and human health. An example is given by Lazar *et al.* who recently showed that there was lower prevalence of the *OCT2* Ser270 allele in patients with essential hypertension compared to general Caucasian populations (103).

II. Research Rationale and Summary of Chapters

Overall research rationale. Interindividual differences in response to xenobiotics, which include many clinically used drugs, are extensive and represent a major problem in rational therapeutics. Such differences in many cases may be caused by genetic differences, the focus of the field of pharmacogenetics. Traditionally, pharmacogenetic studies have begun with a known phenotype followed by discovery of the variant protein and gene responsible for the phenotype, i.e. phenotype-to-genotype studies (104, 105). With the sequence of the human genome and development of high-throughput genotype methods, it is now possible to carry out pharmacogenetic studies in the reverse manner, i.e., genotype-to-phenotype studies. By identifying important drug response genes, identifying the variants in the genes, and then studying the response to drugs in individuals who carry the variants, we can determine whether there is a genetic basis for variation in drug response and learn about each variant or haplotype of a particular drug response gene.

Drug response is determined by both pharmacokinetics and pharmacodynamics. Until recently, pharmacokinetic studies have focused largely on drug metabolizing enzymes. However, it is becoming increasingly clear that membrane transporters are also major determinants of pharmacokinetics (1-3). For example, the efflux transporter, P-glycoprotein, plays a role in drug absorption and distribution (5, 106). Influx transporters, such as organic anion transporting polypeptide 2 (OATP2), encoded by *SLCO1B1*, also appear to be related to pharmacokinetics of many drugs (107-109).

By affecting pharmacokinetics and thus the drug concentrations in the plasma and tissues, the function of a drug transporter may be an indirect determinant of pharmacodyanamics. Alternatively, in theory, by controlling drug concentrations in target tissues and in the vicinity of receptors and other drug targets, membrane transporters may be more direct determinants of pharmacodynamics. Currently, there have been few studies demonstrating the relationships between transporters and pharmacologic effects.

The overall research goal for this dissertation is to determine the physiological and pharmacological roles of OCT1 and to determine the effect of genetic variation in OCT1 on response to drugs. Specifically, this dissertation focuses on the transporter OCT1 and its substrate metformin, a widely used antidiabetic agent. Studies include genetic studies, functional genomic studies in cells, and studies in mice and humans.

The detailed review of OCTs, including OCT1, has been presented in Section I of this introductory chapter. Below, a brief background of metformin is presented.

Background of metformin. The biguanide metformin is widely used as a first-line therapy for the treatment of type 2 diabetes (16). Recently, metformin has also been implicated in the treatment/prevention of fatty liver diseases and polycystic ovary syndrome (110, 111). However, despite its extensive clinical usage, the mechanisms underlying its systemic disposition and pharmacologic effects are not clear.

The clinical pharmacokinetics of metformin has been well studied and reviewed (112). In humans, metformin has an oral bioavailability of 40 to 60%, and the absorption in the intestine is apparently complete within six hours of ingestion (113). It seems that intestinal absorption of metformin involves an active, saturable yet unidentified transporter mechanism, as evidenced by the inverse relationship between dose and bioavailability. Metformin does not bind to plasma proteins and is rapidly distributed after absorption with a volume of distribution ranging from 63 to 280 liters. The drug is eliminated from the body exclusively in the kidney without any significant metabolism. Elimination half-life is thought to reflect the slow intestinal absorption of metformin. Renal and total clearances are reported to range from 20 to 27 and 26 to 42 liter/hour, respectively. The pharmacokinetic profile of metformin is best described by a two-compartment open model. The plasma elimination half-life after oral administration in healthy volunteers ranges from 2.0 to 6.0 hours. Only a few clinical drug-drug interactions have been reported for metformin. These include coadministration of metformin with iodine preparations (114) and cimetidine (115), both of which decrease metformin renal excretion, and guar gum (116), which reduces metformin absorption. Overall, there is considerable variability in metformin pharmacokinetics.

Metformin is usually a safe drug with side effects most frequently from gastrointestinal discomfort such as nausea, abdominal pain, and diarrhea. Lactic acidosis is a rare but severe, dangerous adverse effect of metformin. It seems that this adverse effect is not necessarily associated with plasma metformin concentrations (117). Metformin appears to ameliorate hyperglycemia by reducing gastrointestinal glucose absorption and hepatic glucose production and by improving peripheral glucose utilization (16). The molecular mechanisms underlying metformin action seem to be related to its activation (phosphorylation) of the so-called "energy-sensor", AMP-dependent kinase (AMPK) (118) (Figure 1.6), which suppresses glucagon-stimulated glucose production (118) and causes an increase in glucose uptake in muscle and in hepatic cells (118, 119), *via* unclear mechanisms. The activation of AMPK may also be responsible for the improvement of lipid metabolism by metformin. Recently, the protein threonine kinase, LKB1, which phosphorylates AMPK, has also been reported to be involved in metformin effects (120). While metformin has target effects in the liver, skeletal muscle, and intestine, both AMPK and LKB1 are ubiquitously expressed (121). A fundamental question is thus raised about which factor(s) determine the tissue specificity of metformin action. Another fundamental question is whether metformin activates AMPK *via* a plasma membrane or an intracellular target. Because the drug is positively charged under physiological pHs, and does not readily cross cell membranes, it is reasonable to propose that the drug activates AMPK *via* a receptor on the plasma

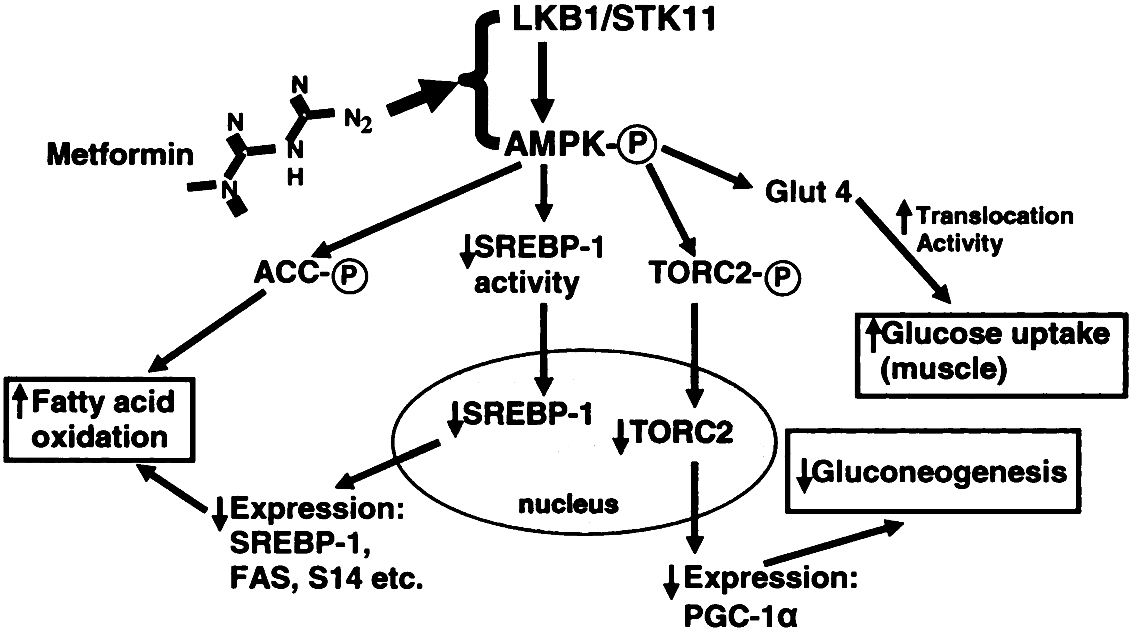

Figure 1.6. Proposed mechanisms of metformin action. Metformin activates (phosphorylates) AMPK, which results in 3 primary effects shown by boxes. Abbreviations: LKB1/STK11, Serine-threonine kinase 11; AMPK, AMP-activated kinase; ACC, acetyl-CoA carboxylase; SREBP-1, Sterol-regulatory element binding protein 1; TORC2, Target of rapamycin complex 2; Glut 4, Glucose transporter 4; FAS, Fatty acid synthase; S14, Spot-14; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator-1α.

membrane. Alternatively, the drug may enter cells *via* a transporter and activate AMPK only after the intracellular entry.

Metformin has been well characterized *in vitro* as a substrate of OCTs (32, 50). Moreover, compared to wild type mice, *Oct1-/-* mice have reduced metformin distribution to the liver and intestine (50). Metformin caused significantly increased blood lactate concentration in wildtype mice compared to only a slight increase in *Oct1-/-* mice (122). These data suggest that OCT1 is involved in metformin disposition and the adverse effect of lactic acidosis. Therefore, by further studying the roles of OCT1 in metformin action, evidence for addressing the two fundamental questions described above is expected. Clinically, there is considerable variability in the disposition of and response to metformin (123, 124). In this dissertation study, the link between genetic variation in human *OCT1* and variation in disposition of metformin and response to the drug is explored.

Summary of chapters. A summary of the work in each data chapter is presented below.

Chapter 2

The goal of the studies in Chapter 2 was to identify genetic variation in human OCT1, encoded by *SLC22A1*, to understand the genetic basis of extensive interindividual differences in xenobiotic disposition. The exons and their flanking intronic regions of OCT1 were resequenced with two hundred and forty-seven DNA samples from individuals of diverse ethnic backgrounds. In particular, we functionally characterized 15 protein-altering variants of OCT1 in *Xenopus* oocytes using the model substrate MPP$^+$. Five variants were found to be reduced or non-functional variants (OCT1-R61C, OCT1-P341L, OCT1-G220V, OCT1-G401S, and OCT1-G465R). Interestingly, all variants that reduced or eliminated function altered evolutionarily conserved amino acid residues. In general, variants with decreased function had amino acid substitutions that resulted in more radical chemical changes (higher Grantham values) and were less evolutionarily favorable (lower Blosum62 values) than variants that maintained function. A variant with increased function (OCT1-S14F) changed an amino acid residue such that the human protein matched the consensus of the OCT1 mammalian orthologs. The results indicate that changes at evolutionarily conserved positions of OCT1 are strong predictors of decreased function and suggest that a combination of evolutionary conservation and chemical change might be a stronger predictor of function. Moreover, the data suggest that genetic variation in *SLC22A1* gene may contribute to interindividual differences in xenobiotic disposition.

Chapter 3

Metformin is among the most widely prescribed drugs for the treatment of type 2 diabetes. The organic cation transporter, OCT1, plays a role in the hepatic uptake of metformin, but its role in the therapeutic effects of the drug, which involve activation of the "energy sensor" AMPK, is unknown. Studies have shown that human OCT1 is highly polymorphic (Chapter 2). In this chapter, we investigated whether OCT1 plays a role in the action of metformin and whether individuals with OCT1 polymorphisms have reduced response to the drug. In mouse hepatocytes, deletion of *Oct1* resulted in a reduction in the effects of metformin on AMPK phosphorylation and gluconeogenesis. In *Oct1*-deficient mice the glucose-lowering effects of metformin were completely abolished. Seven non-synonymous polymorphisms of OCT1 that exhibited reduced uptake of metformin were identified. Notably, OCT1-420del (allele frequency of ca. 20% in European Americans), previously shown to have normal activity for model substrates (Chapter 2), had reduced activity for metformin. In clinical studies, the effects of metformin on glucose tolerance tests were significantly lower in individuals carrying reduced function polymorphisms of OCT1. Collectively, the data indicate that OCT1 is required for metformin therapeutic action and that genetic variation in OCT1 may contribute to variation in response to the drug.

Chapter 4

The goal of studies in this chapter was to further determine whether OCT1 function has an effect on the pharmacokinetics and pharmacodynamics of metformin *in vivo* in animals and humans. The pharmacokinetics of oral metformin was compared between *Oct1* knockout mice (*Oct1-/-*) and wild-type mice. Glucose tolerance tests (GTT) were conducted in the two genotypes of mice to study the pharmacodynamics of metformin. Furthermore, metformin pharmacokinetics and the plasma levels of insulin, lactate, cholesterol and triglycerides before and after a short-term metformin treatment (two doses of 1850 mg in 12 hours) were compared between healthy volunteers who carried a decreased function OCT1 variant and those who carried OCT1 reference alleles. *Oct1* deficiency only had a marginal effect on oral metformin pharmacokinetics in mice, with trends of higher AUC, higher plasma concentrations, and lower oral volume of distribution in *Oct1-/-* mice in comparison to wild-type control mice. However, metformin improved glucose tolerance in the wild-type mice, with no effect in the *Oct1-/-* mice. Surprisingly, there were significant differences in metformin pharmacokinetics between healthy volunteers who carried a decreased function OCT1 variant and those who carried OCT1 reference alleles. In addition, the attenuation of insulin secretion by metformin was significantly impaired in individuals who carried the variants compared to those who only carried reference alleles. The data indicated that OCT1 function affects the pharmacokinetics and pharmacodynamics of metformin. The findings may have clinical implications not only for metformin itself but also for other clinically used drugs that interact with OCT1.

Chapter 5

OCT1 has been well recognized as a transporter for endogenous compounds and xenobiotics. However, the physiological roles of this protein remain unclear. In an attempt to study the role of OCT1 in metformin therapy on fatty liver disease, we generated *Oct1* knockout (*Oct1-/-*) and leptin deficient (*ob/ob*) double mutant mice (*Oct1-/-, ob/ob*). Surprisingly, the double mutant mice had markedly reduced fatty liver in comparison to *Oct1+/+*, *ob/ob* mice which are disease models of fatty liver, insulin resistance and diabetes. The goal of studies in this chapter was to follow this unexpected observation and to understand whether OCT1 is involved in lipid and glucose metabolism and in pathogenesis of fatty liver. The effects of OCT1 on hepatic triglyceride homeostasis, peripheral adiposity, and plasma biochemistry were studied in the mice of different *Oct1* and *leptin* genotypes. *hOCT1*-transgenic *C. elegans* were also studied with respect to their intestinal fat phenotype. In addition, the role of AMP-activated kinase (AMPK) in the improvement of fatty liver by *Oct1* deficiency was explored. The data suggest that *Oct1* deficiency improved fatty liver in *leptin*-deficient obese mice and had effects on the homeostasis of nutrients such as triglyceride and glucose. Therefore, in addition to its function as a drug transporter, OCT1 may have physiological roles in nutrient homeostasis. Identification of molecular events in the regulation of nutrient metabolism by Oct1 might bring further insight into the mechanisms of development of metabolic syndromes such as fatty liver, insulin resistance, and diabetes. This study warrants further studies to characterize the observed phenotypes in more detail and to decipher the underlying mechanisms.

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CHAPTER 2

EVOLUTIONARY CONSERVATION PREDICTS FUNCTION OF VARIANTS OF THE HUMAN ORGANIC CATION TRANSPORTER, OCT1[1]

Introduction

Interindividual differences in response to xenobiotics, which include many clinically used drugs, are extensive and represent a major problem in rational therapeutics. Such differences in many cases may be caused by inherited differences in enzymes and transporters which function in drug elimination in the liver (1). The organic cation transporter, OCT1, is a major transporter located in the sinusoidal membrane of the liver that mediates the uptake of many organic cations from the blood into hepatocytes. These organic cations include clinically used drugs (e.g., metformin), endogenous compounds (e.g., dopamine), as well as toxic substances (e.g., MPP^+) (2-6). Although rare mutations in liver transporters (e.g., MRP2 and BSEP) have been associated with Mendelian diseases such as Dubin–Johnson syndrome (7-9), little is known about the contribution of common variants of these transporters to variation in hepatic drug disposition and disease.

To understand the genetic basis of extensive interindividual differences in xenobiotic disposition, we have screened for variants in 24 different membrane transporters, including OCT1, in 247 ethnically diverse DNA samples (10). Genetic variants of OCT1 identified in the screening study include 14 nonsynonymous single-

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[1] This chapter was published in a manuscript entitled: Evolutionary conservation predicts function of variants of the human organic cation transporter, OCT1. 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; Pharmacogenetics Of Membrane Transporters Investigators. *Proc Natl Acad Sci U S A* 100:5902-5907. I thank all other authors who made the contribution to this work.

nucleotide polymorphisms (SNPs), and one 3-bp deletion that leads to deletion of a methionine residue. In related work in our laboratory, we used allele frequency distributions to assess two predictors of function, evolutionary conservation among close orthologs and chemical relatedness (10). Here we experimentally evaluate these predictors by characterizing the function of the 15 protein-altering variants of OCT1.

Materials and Methods

Construction of Variants and Functional Characterization in oocytes. OCT1 cDNA with the reference sequence (GenBank accession nos. U77086 and NM_003057) was subcloned into expression vectors pEXO and pEGFP. The Stratagene QuikChange site-directed mutagenesis kit was used to construct mutant cDNA following the manufacturer's protocols. The variants were confirmed by DNA sequencing. Healthy stage V and VI *Xenopus laevis* oocytes were injected with 50 nl of diethylpyrocarbonate (DEPC)-treated water or 25 ng of capped cRNA transcribed *in vitro* with T7 RNA polymerase (mCAP RNA Capping kit; Stratagene) from *SpeI*-linearized pEXO plasmids containing the reference or mutant OCT1 cDNA inserts. Before injection, an aliquot of the cRNA was run on an agarose gel to verify that it was not degraded; RNA concentrations were determined by spectrophotometry.

The injected oocytes were maintained in modified Barth's solution at 18°C before uptake studies. Uptake experiments with the injected oocytes were conducted 2 – 4 days after injection as described (4). Groups of seven to nine oocytes were incubated in buffer containing 0.1 μM ^{3}H-MPP$^+$ (78 Ci/mmol; 1Ci = 37 GBq) at room temperature for 1 h. Uptake was stopped by washing the oocytes five times with 3 ml of ice-cold Na$^+$ buffer.

Oocytes were then lysed individually with 100 µl of 10% SDS, and the radioactivity associated with each oocyte determined. For kinetic and inhibition studies, unlabeled substrate or inhibitors were added to the incubation mixture as needed. Each data point was determined in duplicate or triplicate for each experiment unless indicated. Data are presented as mean ± standard deviation unless indicated. All experiments were repeated at least once using different batches of oocytes or different cell passages. Water-injected oocytes and oocytes injected with reference OCT1 RNA served as controls within each batch of oocytes.

Cell Culture and Stable Transfection. Madin – Darby canine kidney cells (passages 10 – 40) were maintained in MEM Eagle's with Earle's balanced salt solution supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% (vol/vol) FBS in 5% CO_2/95% air. Cells were transfected with pEGFP plasmids containing the reference or mutant hOCT1 cDNA inserts, or empty vector by Effectene Transfection Reagent following the manufacture's protocols (Qiagen, Valencia, CA). Three days after transfection, stable clones were selected in media containing 0.7 mg/ml G418. After 10 – 14 days, individual stable clones were isolated and positive clones were further selected by immunocytochemistry. For subsequent transport studies, cells were polarized by growth on Transwell filters (0.4-µm pore size, 12-well plate, Costar, Cambridge,MA) at a confluent density for 7 days with regular media changes as described elsewhere (11).

Confocal Microscopy. Madin – Darby canine kidney cells grown on filters for 7 days as stated above were fixed with 4% paraformaldehyde, permeablized with 0.025

(wt/vol) saponin in PBS, stained with Texas-red-conjugated phalloidin for visualization of actin, and mounted on slides in Vectashield mounting medium. Samples were analyzed by using a Bio-Rad MRC-1024 confocal microscope.

Results and Discussion

Functional Activity of hOCT1 Variants. The 15 protein variants identified by Leabman *et al.* (10) in an ethnically diverse sample have changes in both loops (nine variants) and transmembrane domains (six variants) of OCT1 (Figure 2.1). Of the 14 substitution variants of OCT1, we observed that five exhibited decreased function (OCT1-R61C, OCT1-G220V, OCT1-P341L, OCT1-G401S, and OCT1-G465R) and one had increased function (OCT1-S14F) (Table 2.1, Figure 2.2). MPP^+ uptake was restored to normal levels after the variant sequences were changed to the reference sequence by site-directed mutagenesis (Figure 2.3), demonstrating that these amino acid changes were responsible for the altered activities. A recent screen for OCT1 variants in a European American population (n = 57) identified three variants with decreased function, OCT1-R61C and OCT1-G401S, which were also found in our study, and OCT1-C88R, a rare variant, which was not (12). The basis for the functional defects was identified for three of the variants. The two variants with reduced function (OCT1-R61C and OCT1-P341L) had an increased K_m (88.7 and 24.7 μM, respectively, compared with 13.9 μM for the reference OCT1; data not shown) and a reduced V_{max} (10.0 pmol per oocyte per hour and 10.4 pmol per oocyte per hour, respectively, compared with 18.4 pmol per oocyte per hour for the reference OCT1; data not shown). The nonfunctional variant, OCT1-G465R, tagged with GFP, exhibited reduced localization at the basolateral surface whereas the

ORGANIC CATION TRANSPORTER 1 (SLC22A1)

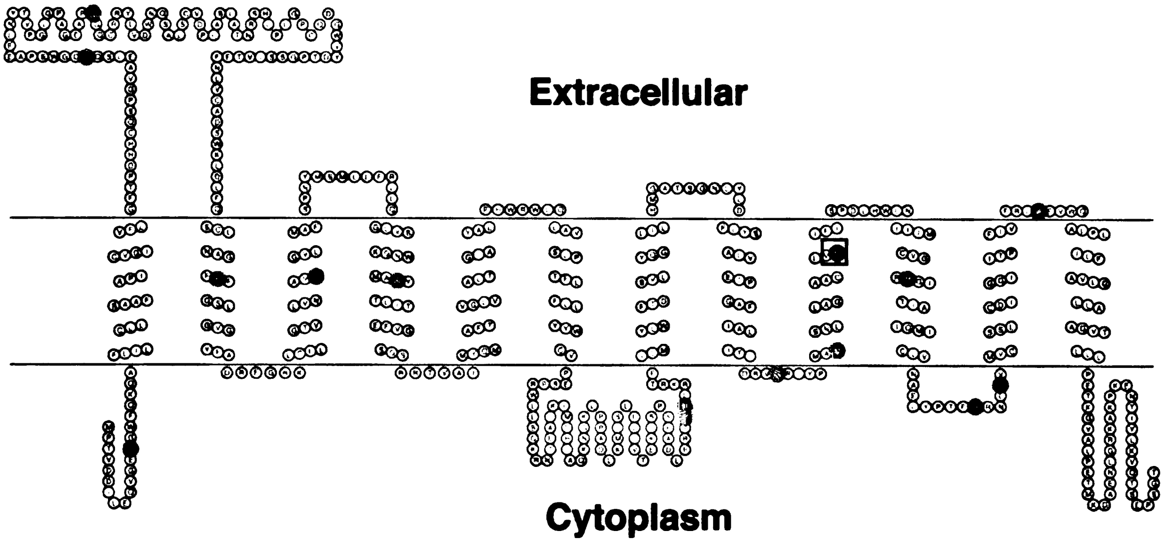

Figure 2.1. Secondary structure of OCT1 with coding region SNPs. The transmembrane topology diagram was rendered using the transmembrane protein display software TOPO [S. J. Johns (University of California, San Francisco) and R. C. Speth (Washington State University, Pullman), available at the University of California, San Francisco Sequence Analysis Consulting Group web site, www.sacs.ucsf.edu/TOPO/topo.html]. Nonsynonymous amino acid changes are shown in red, and amino acid deletion is shown in blue in a box.

Table 2.

Amino Acid Change*
S14F
R61C
L85F
F160L
S189L
G220V
P341L
R342H
G401S
M408V
M420Del
M440I
V461I
G465R
R488M

NA, not av

*Positions
accession
MPP⁺ upta

†Some sam
actual DN
American;
chromoson

‡++, increa
+/–, reduc

§Grantham
values indi
(evolutiona
mammalian
less accepta
from 0 to 1.
substitutions

¶SIFT score

Table 2.1. Frequencies and characteristics of 15 protein-altering variants in OCT1

Amino	Allele frequency†							Scoring systems for nonsynonymous variants§			
Acid	Total	AA	EA	AS	ME	PA					
Change*	(n = 494)	(n = 200)	(n = 200)	(n = 60)	(n = 20)	(n = 14)	Function‡	Grantham	EC/EU	BLOSUM62	SIFT¶
S14F	0.013	0.031	0	0	0	0	+ +	155	EU	-2	1.00
R61C	0.031	0	0.072	0	0.056	0	+/-	180	EC	-3	0.00
L85F	0.004	0.01	0	0	0	0	+	22	EU	0	0.00
F160L	0.032	0.005	0.065	0.017	0.05	0	+	22	EC	0	0.00
S189L	0.002	0	0.005	0	0	0	+	145	EC	-2	0.00
G220V	0.002	0.005	0	0	0	0	-	109	EC	-3	0.00
P341L	0.047	0.082	0	0.117	0	0	+/-	98	EC	-3	0.00
R342H	0.012	0.031	0	0	0	0	+	29	EU	0	0.16
G401S	0.008	0.007	0.011	0	0	0	-	56	EC	0	0.00
M408V	0.682	0.735	0.598	0.762	0.786	0.929	+	21	EU	1	0.08
M420Del	0.105	0.029	0.185	0	0.214	0	+	NA	NA	NA	NA
M440I	0.002	0.005	0	0	0	0	+	10	EC	1	0.00
V461I	0.004	0.01	0	0	0	0	+	29	EU	3	1.00
G465R	0.016	0	0.04	0	0	0	-	125	EC	-2	0.00
R488M	0.02	0.05	0	0	0	0	+	91	EU	-1	0.35

NA, not available.

*Positions are relative to the ATG start site and are based on the cDNA sequence from GenBank accession no. NM_003057. Changes shown in bold result in decreased function (as measured by MPP$^+$ uptake) compared to the reference OCT1.

†Some samples contained amplicons that could not be sequenced. Allele frequencies were based on actual DNA samples sequenced. Total, entire sample; AA, African American; EA, European American; AS, Asian American; ME, Mexican American; PA, Pacific Islander; n is the number of chromosomes.

‡++, increased function relative to the reference OCT1; +, function similar to that of reference OCT1; +/– , reduced function; – , no function.

§Grantham values range from 5 to 215, in which low values indicate chemical similarity and high values indicate radical differences; EC/EU indicates classification of nonsynonymous variants as EC (evolutionarily conserved) or EU (evolutionarily unconserved) based on sequence alignments with mammalian orthologs (10); BLOSUM62 values range from – 4 to +3, where negative values indicate less acceptable and non-negative values indicate more acceptable substitutions; SIFT values range from 0 to 1, where values close to 0 represent less tolerated and those near 1 represent more tolerated substitutions.

¶SIFT scores were assigned as described (10).

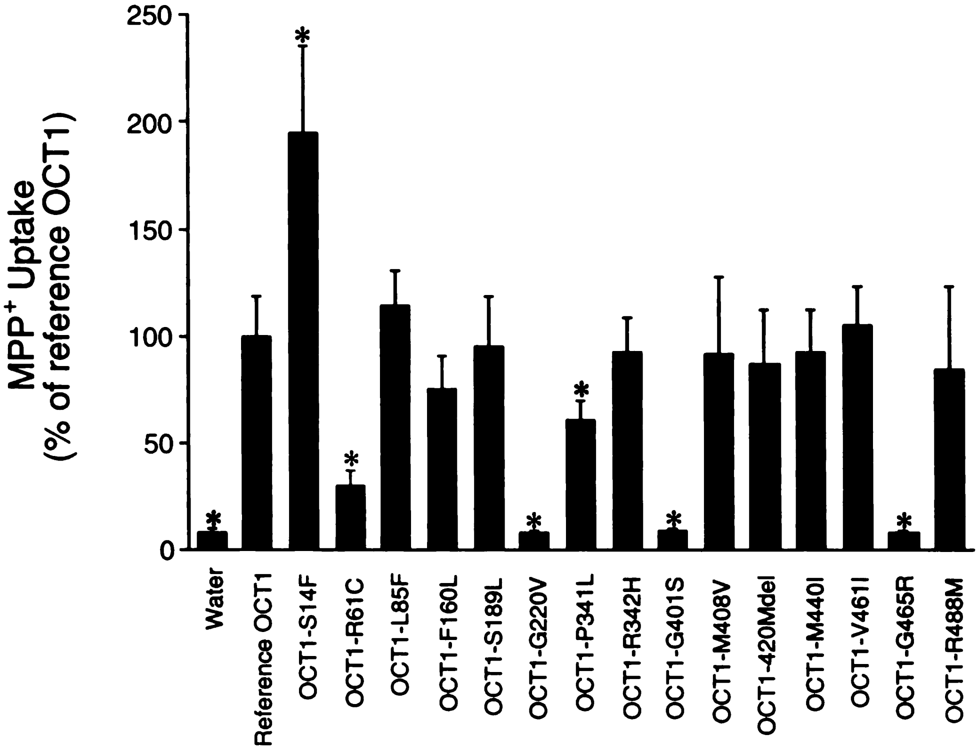

Figure 2.2. Functional characterization of natural variants of OCT1. Uptake of MPP^+ was conducted in oocytes expressing the reference OCT1 and each of the 15 protein altering OCT1 variants. *X. laevis* oocytes were injected with ≈50 ng of RNA. Uptake of MPP^+ (0.9 μM unlabeled MPP^+, 0.1 μM $^3H\text{-}MPP^+$) was measured at room temperature after incubation for 1 h. Data is representative of experiments carried out with three different batches of oocytes. Each value represents mean ± SD from seven to nine oocytes. *$P < 0.01$ compared to Reference OCT1.

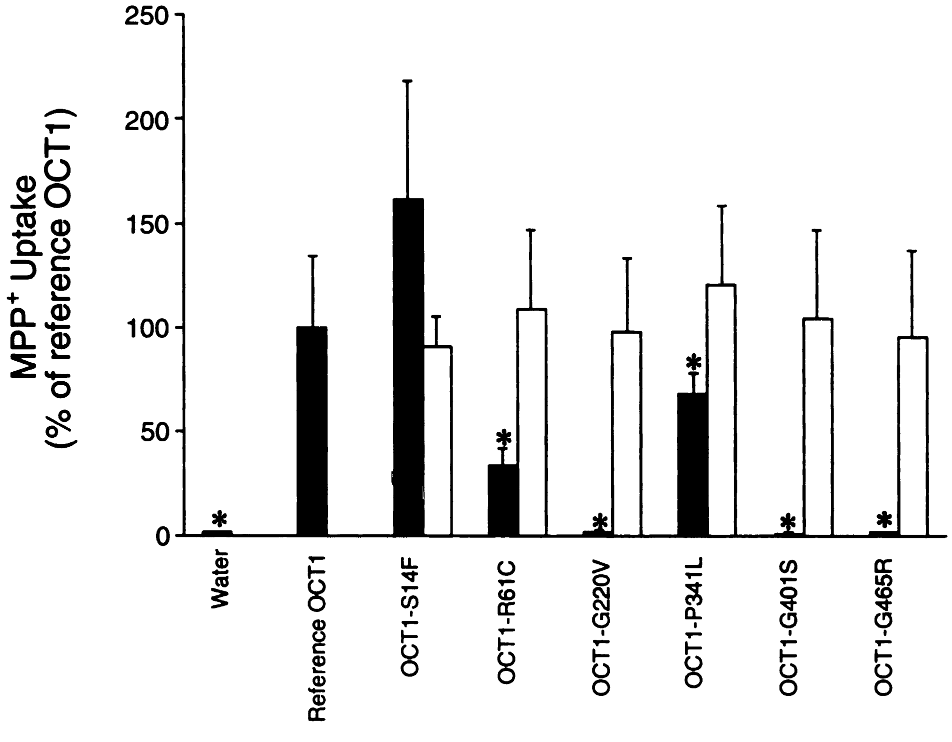

Figure 2.3. Recovery of function for six OCT1 variants with reduced or increased function. Dark-shaded columns represent uptake by variants; lighter-shaded columns represent uptake by variants changed to the OCT1 reference sequence at a single nucleotide position by site-directed mutagenesis. Uptake of MPP^+ was conducted as described in the legend of Figure 2.2.

reference OCT1 and OCT1-R488M, a variant that retained function, were localized to the basolateral membrane (Figure 2.4) (13). It is striking that all three of the nonfunctional variants altered evolutionarily conserved glycine residues, suggesting that these residues in OCT1 may be particularly important for function (Figure 2.5). Two of them (at positions 401 and 465) are present in paralogs, OCT2 and OCT3; the other (at position 220) is present in OCT2 only, further suggesting the importance of these glycine residues in function of organic cation transporters.

We next examined the phenotype of haplotypes containing two nonsynonymous changes and measured their MPP^+ uptake (Figure 2.6). M408V had an allele frequency of 68.2% in the 494 chromosomes and often occurred with other nonsynonymous mutations in haplotypes identified in this study (14). Thus, the phenotype of variants with two alterations (M408V and each of the other 14 amino acid changes) was examined. These doubly altered variants exhibited phenotypes like those of the 14 variants with the single amino acid changes; in other words, M408V did not alter function. We also examined the activity of a variant OCT1-F160L G401S, which occurs in a predicted haplotype. Like OCT1-G401S, OCT1-F160L G401S exhibited no MPP^+ uptake.

Strikingly, OCT1-S14F displayed increased MPP^+ uptake (Figure 2.2) which was reversed when the variant phenylalanine was restored to serine. Kinetic studies revealed that OCT1-S14F had a somewhat lower K_m (8.2 μM) and higher V_{max} (25.5 pmol/oocyte/hr) than the reference OCT1 (although the difference was not statistically significant). The ratio of V_{max} to K_m for OCT1-S14F was ≈2-fold greater than for the reference. Amino acid variants with increased activity are observed infrequently but are of biological interest because they provide information about residues that govern protein

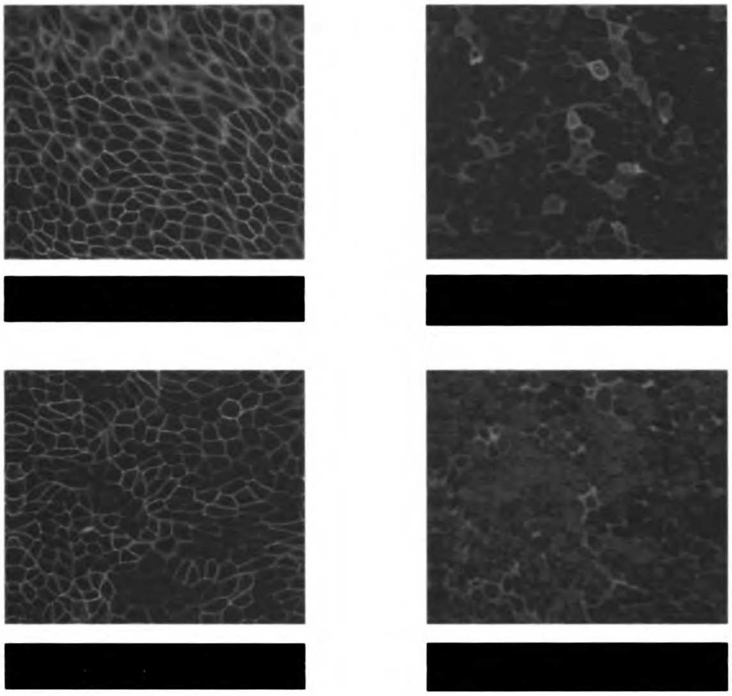

Figure 2.4. Localization of GFP-tagged reference OCT1, OCT1-G465R, and OCT1-R488M in Madin – Darby canine kidney (MDCK) cells. MDCK cells were transfected with plasmids eGFP-OCT1, eGFP-OCT1-G465R, eGFP-OCT1-R488M, and eGFP. Stable clones were selected with G418. Cells were polarized by growth on filters for 7 days. The cells were fixed, permeabilized, stained for actin with Texas-red-conjugated X phalloidin, and visualized by confocal fluorescence microscopy. A horizontal section (*Upper*) and a vertical section with the apical membrane on top (*Lower*) are shown for each set of transfected cells. (*Upper Left*) eGFP-OCT1. (*Upper Right*) eGFP-OCT1-G465R. (*Lower Left*) eGFP-OCT1-R488M. (*Lower Right*) eGFP.

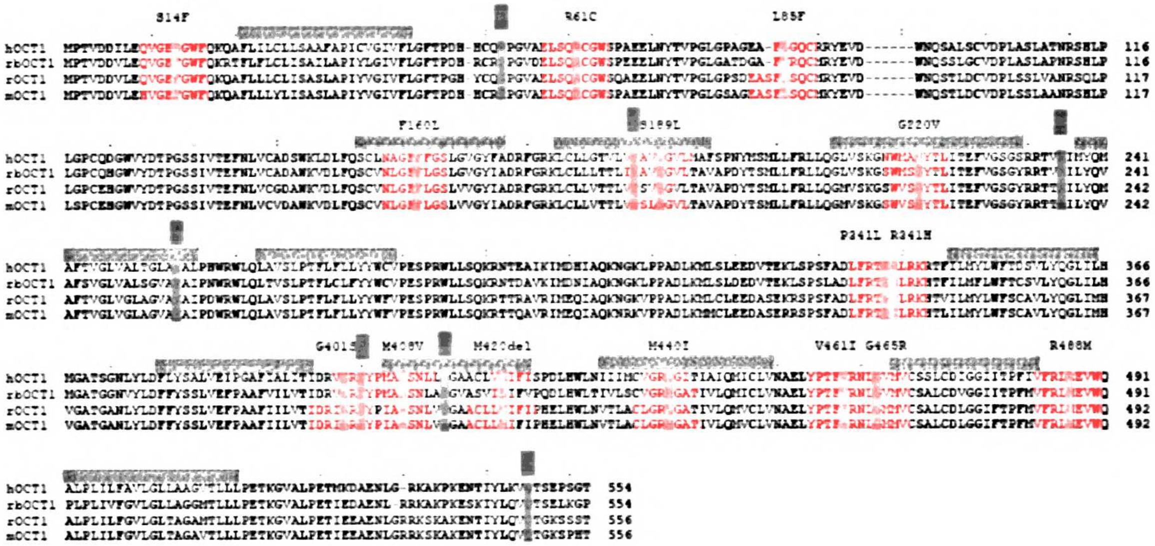

Figure 2.5. Alignment with mammalian orthologs. Human OCT1 is aligned with rbOCT1, rOCT1, and mOCT1. The variants identified from OCT1 coding sequence in this study are shown above the sequence, and their corresponding sequence alignment are highlighted. For nonsynonymous variants, pure red shading indicates 100% sequence conservation and yellow indicates only one amino acid difference. Synonymous variants and their corresponding amino acid positions are indicated as green. The transmembrane domains (TMDs) of human OCT1, as predicted previously (4), are also shown above the sequence as blue bars.

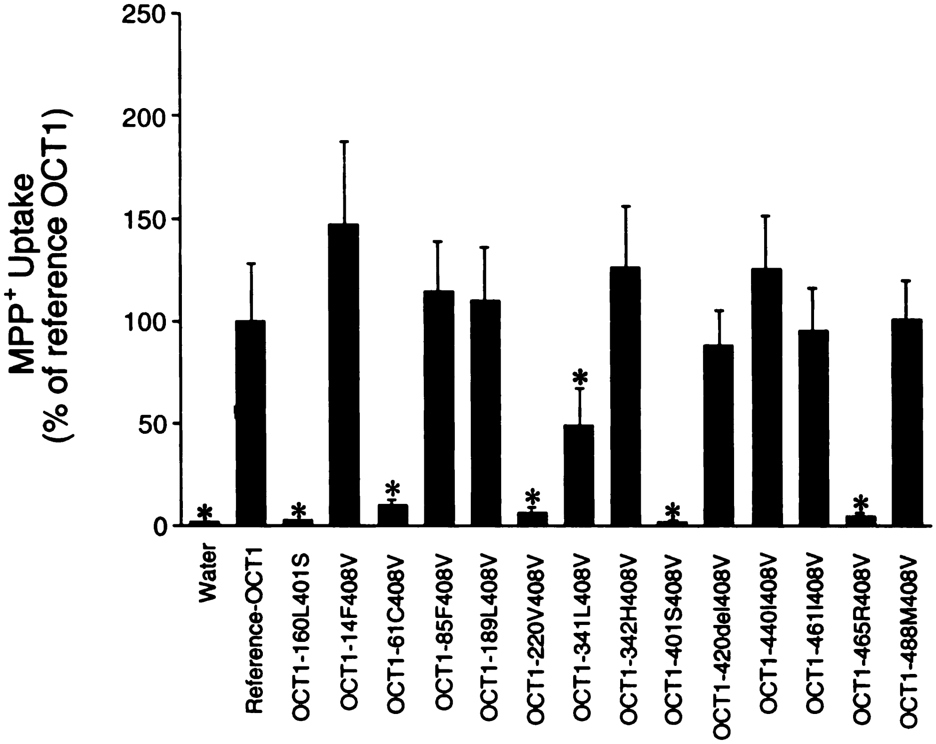

Figure 2.6. Effect of double mutations in OCT1 on transporter function. Uptake of MPP$^+$ was conducted as described in the legend of Figure 2.2. *$P < 0.01$ compared to Reference OCT1.

activity and perhaps specificity. The phenylalanine residue at codon 14 is highly conserved among mammalian orthologs of OCT1, including rat, rabbit, mouse, and chimpanzee (Figure 2.5), and is also found at the corresponding position in human OCT2 and OCT3. The human S14F variant, found exclusively in the African American population sample (at 3.1% allele frequency), restores evolutionary conservation at this position. Prior functional studies with human OCT1 have shown that it has lower transport activity than rat, rabbit, and mouse orthologs (15). Whether the phenylalanine residue in the rat, rabbit or mouse OCT1 that corresponds to human position 14 contributes to their increased activity relative to human OCT1 remains to be determined. These observations suggest that high OCT1 activity may be less optimal for fitness of humans than for other mammals. Of the 155 nonsynonymous variants identified by Leabman *et al.*, a total of eight in addition to OCT1-S14F changed the human protein to match the consensus of the other mammalian orthologs (10). It will be interesting to see whether they also affect transporter activity, in particular, whether they increase activity.

Prediction of Function by Evolutionary Conservation and Chemical Change. One important reason for developing algorithms and criteria to predict the function of nonsynonymous variants is that it informs the choice of which SNPs to choose for genetic association studies. We have therefore evaluated the changes in OCT1 variants by different criteria (chemical change, evolutionary conservation, and amino acid substitution scoring matrices such as BLOSUM62 and SIFT) (16, 17). In general, the variants with decreased function had larger chemical changes (greater Grantham values) than variants that did not reduce function (Table 2.1; Figure 2.7A), suggesting that the

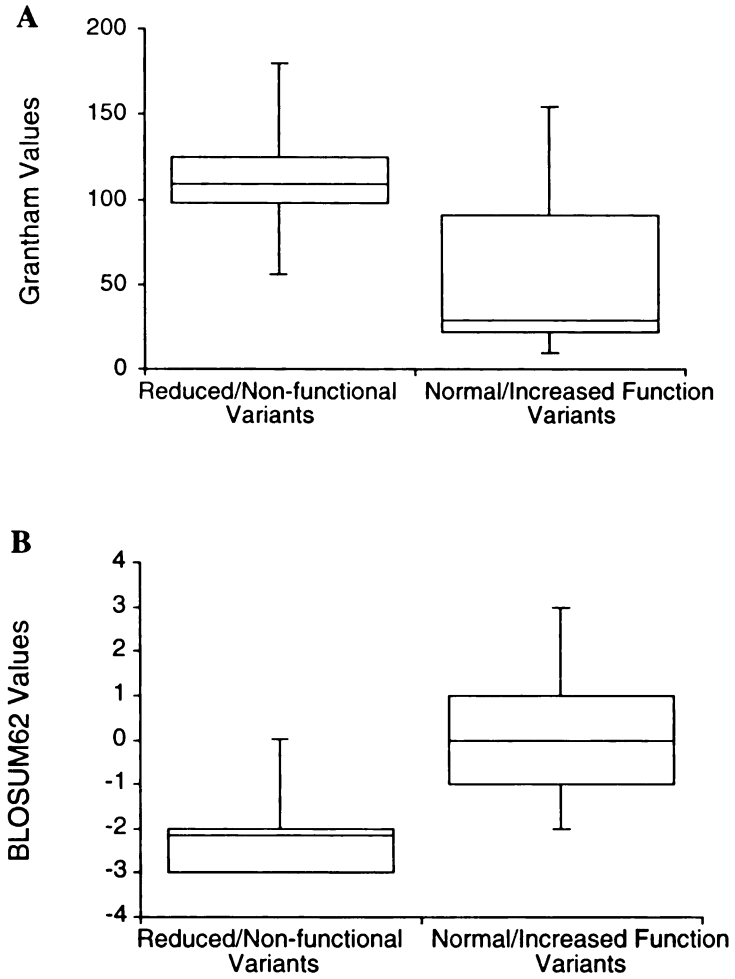

Figure 2.7. Grantham and BLOSUM62 values for OCT1 variants. *(A)* Grantham values for OCT1 variants with or without decreased function. Mean ± SE values for the variants with decreased function are 114 ± 20 *versus* 58.2 ± 19 for the variants with increased or normal function, respectively ($P < 0.1$). *(B)* BLOSUM62 values for OCT1 variants with or without decreased function. Mean ± SE values for the variants with decreased function are − 2.2 ± 0.57 *versus* 0.0 ± 0.53 for the variants with increased or normal function, respectively ($P < 0.02$).

nature of the amino acid change may be a useful predictor of function (18). For example, of the seven variants with Grantham values ≤ 56, six (with values from 10 – 29) exhibited normal function, whereas one (with a value of 56) was nonfunctional. The usefulness of Grantham values to predict function was weaker for more radical amino acid substitutions. Of the seven variants with Grantham values > 90, four (with values from 98 –180) exhibited reduced function, two (with values of 91 and 145) exhibited normal function, and one (with a value of 155) exhibited increased function. The three completely nonfunctional variants, which have substitutions of glycine residues, had Grantham values of 109 (G220V), 56 (G401S), and 125 (G465R). We constructed a variant with a more modest chemical change at position 465, OCT1-G465A (GV 61) and observed that it exhibited normal activity (data not shown), indicating that radical change at this position is responsible for reduced function. The evolutionary conservation of G401 and the low Grantham value of the G401S variant suggest that this position has a particularly stringent requirement for glycine.

The analysis presented by Leabman *et al.* showed that evolutionary conservation is a strong predictor of allele frequency, indicating that substitutions at evolutionarily conserved (EC) positions are more deleterious than those at evolutionarily unconserved (EU) positions (10). Our analysis of the 14 amino acid substitution variants of OCT1 made it possible to determine whether this prediction is experimentally validated. As before, we defined EC residues as those that were identical in all members of a set of mammalian OCT1 orthologs; EU residues are those in which there is not a consensus (10). Five of eight variants that affected evolutionarily conserved residues exhibited decreased function whereas none of the six variants that altered evolutionarily unconserved residues decreased function (Table 1, $X^2 = 5.83$, $P < 0.05$).

BLOSUM62, an amino acid substitution matrix, is derived from amino acid changes in an unselected protein set (17) and has been used to infer protein function (19). BLOSUM62 scores of nonsynonymous SNPs did not predict allele frequency distribution and therefore were not indicators of function for the set of 24 transporters taken as a whole (10). To experimentally determine whether BLOSUM62 values predicted function for OCT1 variants, we compared BLOSUM62 values for the variants with decreased function with those for the variants that retained function. We observed that the values for the decreased-function variants were significantly more negative (evolutionarily unfavorable) than values for the variants that retained function (-2.2 ± 0.57 *versus* 0.0 ± 0.53, $P < 0.02$) (Figure 2.7B). In particular, six of seven variants with non-negative BLOSUM62 scores (deemed evolutionarily acceptable) exhibited normal OCT1 function. The relationship between negative BLOSUM62 scores was less apparent: four of seven variants with negative BLOSUM62 scores exhibited reduced activity, two exhibited normal activity, and one exhibited increased activity. Overall, these observations on OCT1 support the use of BLOSUM62 scores to predict protein function (10, 19).

SIFT is an algorithm that assigns scores to amino acid changes using alignments of orthologs of the protein of interest (16). Because SIFT incorporates phylogenetic information specific to OCT1, we anticipated that it might be a particularly strong predictor of function of the OCT1 variants. We did not, however, detect a significant difference in the SIFT scores of the variants with decreased function and the variants that retained function. The poor correlation between SIFT score and function resulted primarily from the fact that three variants with alterations at evolutionarily conserved positions, which were assigned a SIFT score of 0 (indicating functional intolerance), exhibited normal function. It is notable that of these three variants, two had only modest chemical changes: OCT1-F160L and OCT1-M440I had Grantham values of 22 and 10, respectively. One other variant (OCT1-L85F) was also assigned a SIFT score of 0 and exhibited normal function. It, too, had a low Grantham value (Grantham value was 22). Leabman *et al.* also noted that alleles present at high frequency that altered evolutionarily conserved residues have low Grantham values, indicating that this change was tolerated even though it affects an evolutionarily conserved residue (10). Our observations on OCT1 reinforce the proposal of Leabman *et al.* that combining evolutionary conservation (e.g., SIFT) with Grantham values may be particularly useful for optimizing algorithms to predict function.

Relationship Between Allele Frequency and Functional Activity of OCT1 Variants. The two most common variants of OCT1, OCT1-M408V and OCT1-M420del (which have allele frequencies >10%; Table 2.1), both exhibited normal function, consistent with the premise that common variants are less likely to exhibit altered function than are rare variants. A similar trend was observed in our recent study of natural variants of OCT2, in which the most common nonsynonymous variant, OCT2-A270S, exhibited transport properties similar to that of the reference OCT2 (20). In contrast, all three nonfunctional variants of OCT1 were present at overall allele frequencies of <2% (Table 2.1). It is worth noting, however, that two of these variants exhibited frequencies of 1.1% and 4% in European Americans and that the two variants with reduced function were present at substantial frequencies, 7.2% in European Americans (R61C) and 8.2% in African Americans (P341L).

Possible Consequences of Variation in OCT1. The high variability of OCT1 may have implications both for human disease and drug response, given that the transporter interacts with a variety of structurally diverse compounds and controls access to drug metabolizing enzymes in the liver (2-6). Genetic variation in OCT1 could also contribute to neurodegenerative diseases because OCT1 appears to govern hepatic uptake and elimination of 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is responsible for a Parkinsonian syndrome (21, 22). Reduced-activity variants of OCT1 may lead to enhanced exposure to endogenous and environmental toxins and contribute to neurodegenerative disease. Variation in OCT1 may influence drug response by altering hepatic drug clearance. Dramatic differences in the liver distribution of the anticancer drug MIBG and the antidiabetic drug metformin have been observed in *Oct1* knockout mice compared with wild-type mice (23, 24). Given that OCT1 is primarily expressed in the liver, we expect to see similar differences between normal individuals and those with variants that have reduced OCT1 function (4, 5).

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CHAPTER 3

THE ORGANIC CATION TRANSPORTER 1, OCT1, IS REQUIRED FOR THE THERAPEUTIC EFFECTS OF METFORMIN[1]

Introduction

Metabolic syndrome and its pathological sequela, type 2 diabetes, have become major health problems in the world. The biguanide metformin is widely used as a first-line therapy for the treatment of type 2 diabetes (1). In addition, the drug has recently been implicated in the treatment and/or prevention of fatty liver diseases and polycystic ovary syndrome (2, 3). Metformin ameliorates hyperglycemia by reducing gastrointestinal glucose absorption and hepatic glucose production and by improving glucose utilization (1). The molecular mechanisms underlying metformin action appear to be related to its activation (phosphorylation) of the so-called "energy-sensor", AMP-dependent kinase (AMPK), which suppresses glucagon-stimulated glucose production and causes an increase in glucose uptake in muscle and in hepatic cells (4, 5). The activation of AMPK may also be responsible for the improvement of lipid metabolism by metformin (4). Recently, the protein threonine kinase, LKB1, which phosphorylates AMPK, has also been reported to be involved in metformin effects (6, 7).

However, despite the extensive clinical usage and the recent research progress, the mechanisms underlying the therapeutic effects of metformin are still not well known.

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[1] This chapter is a manuscript entitled: The Organic Cation Transporter 1, OCT1, is Required for the Therapeutic Effects of Metformin. Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, Yue L, Lo J, Burchard EG, Brett CM, Giacomini KM. Currently revising with *the Journal of Clinical Investigation*. I thank all other authors who made the contribution to this work.

In particular, while metformin is very hydrophilic and positively-charged under physiological conditions due to the strongly basic character of its polar guanidine moiety, it is not known whether a transporter protein that carries metformin across the membrane is required for the therapeutic effects of the drug. Metformin has been well characterized *in vitro* as a substrate of organic cation transporters (OCTs, according to HUGO and MGI gene nomenclature committees, uppercase letters are used throughout the text for the general nomenclature of a transporter and the human homolog, and the rodent homologs begin with an uppercase letter followed by all lowercase letters) including the liver-specific OCT1 and its paralog, OCT2, a transporter expressed in abundance in the kidney (8-11). Compared to wild type mice, *Oct1-/-* mice have reduced metformin distribution to the liver (11); however, it is not known whether this reduced uptake corresponds to a reduction in the therapeutic effects of metformin that occur following AMPK activation including lowering blood glucose levels.

Genetic polymorphisms in drug transporter genes have been increasingly recognized as a possible mechanism accounting for variation in drug response (12). In previous studies, we and others showed that human OCT1 is highly polymorphic in ethnically diverse populations (13-15). Using model substrates, it was shown that a number of non-synonymous polymorphisms of OCT1 exhibit reduced activity in cellular assays. However, the clinical significance of the OCT1 variants has not been investigated. Since response to metformin is clinically variable (16, 17), it is possible that polymorphisms in OCT1 contribute to this variation.

In this study we tested the hypothesis that the cellular uptake of metformin represents the first step in its activation of AMPK. In particular, as the liver is a primary

target of metformin action (1), we hypothesized that Oct1 is required for the therapeutic effects of metformin. Furthermore, we examined the effects of non-synonymous OCT1 polymorphisms on metformin uptake and response in cellular assays. Finally the clinical effects of metformin on oral glucose tolerance tests (OGTT) were compared between individuals with and those without reduced function variants of OCT1.

Materials and Methods

Cell lines and transfection. Clone 9 cells and 3T3-L1 cells were obtained from American Type Culture Collection (Manassas, VA). Human embryonic kidney (HEK) cells (Flp-In-293) were from Invitrogen (Carlsbad, CA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) of high glucose supplemented with 10% FBS, 100 Units/ml penicillin and 100 μg/ml streptomycin (Cell Culture Facility, University of California San Francisco, San Francisco, CA). HEK293 cells were transfected with pcDNA5/FRT vector (Invitrogen) containing the reference (HEK-OCT1), mutant human OCT1 cDNA inserts, or empty vector using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's protocols. The OCT1 variants were identified in a previous study (13) and constructed by site-directed mutagenesis from the reference OCT1 cDNA that was cloned into the pcDNA5/FRT vector. The variants were confirmed through DNA sequencing. After initial transfection, the stable clones were selected and maintained with 75 μg/ml of hygromycin B (Invitrogen). All the cells were grown at 37^0C in a humidified atmosphere with 5% CO_2/95% air.

Generation of *Oct1-/-* mice. Our strategy results in the complete deletion of exons 3-5 and partial deletion of exons 2 and 6 from the murine *Oct1* gene (Figure 3.1). The deleted region is replaced with an IRES-LacZ expression cassette and a positive selection cassette containing the neomycin phophotransferase gene driven by the PGK promoter. Homology arms (5' 3kb and 3' 3.5kb) were cloned by proof-reading PCR from the E14.1 ES cell line and placed on either side of the IRES-lacZ expression cassette and positive selection cassette to generate the targeting construct. Homologous recombination in neomycin resistant ES cells was confirmed by Southern blot of Spe I digested genomic DNA using a 3' external probe which detects 8 kb and 6 kb bands at the wild-type and targeted locus respectively. Approximately one in sixty G418 resistant clones had undergone homologous recombination. Homologous recombination at the 5' end was confirmed in these ES cell clones by Southern blot and both ends were reconfirmed by PCR using primers external to the targeting construct. Gene targeting was performed in E14.1 ES cells. Three targeted clones were injected into C57Bl6/J-derived blastocysts. Male chimaeras were crossed with C57Bl6/J females to produce N1F0 offspring, which were subsequently inter-crossed to generate [C57Bl6/J x 129Ola] N1F1 generation used in initial testing. The mutant mice were crossed with wild-type FBV/N mice for four generations to acquire the FVB/N background in this study.

All animals were housed in a virus-free facility on a 12-hour light-dark cycle. We fed the mice either a standard mouse food or a high-fat diet (diet D12492, Research Diets, New Brunswick, NJ). All experiments on mice were approved by the Institutional Animal Care and Use Committee of University of California at San Francisco (UCSF).

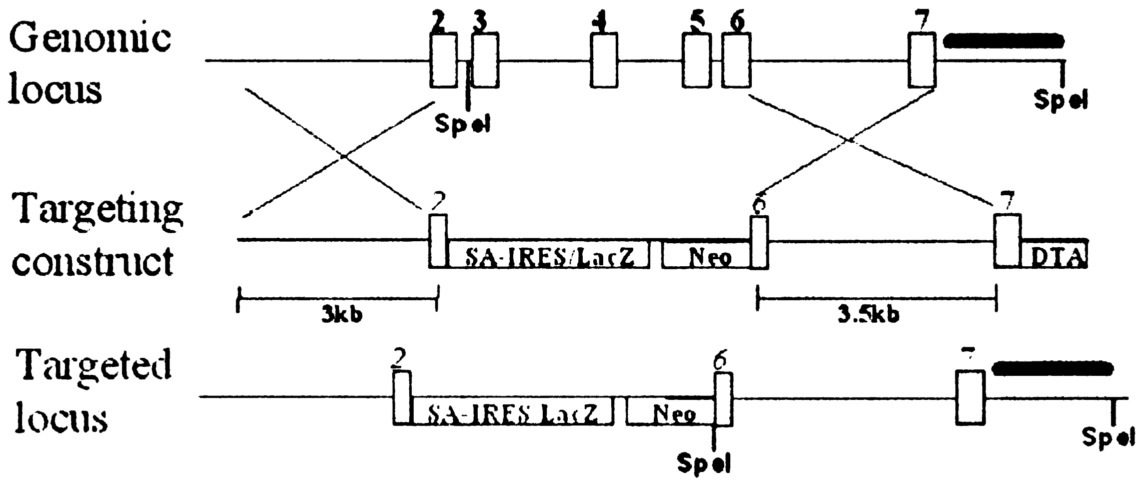

Figure 3.1. Targeted disruption of the *Oct1* gene by homologous recombination. The exons 2 - 7 are shown. The strategy results in the complete deletion of exons 3-5 and partial deletion of exons 2 and 6. Homology arms (5' 3kb and 3' 3.5kb respectively) were cloned by proof-reading PCR and placed either side of the IRES-lacZ expression cassette and positive selection cassette to generate the targeting construct. The deleted region is replaced with an IRES-LacZ expression cassette and a positive selection cassette containing the neomycin phophotransferase gene driven by the PGK promoter. Homologous recombination in neomycin resistant ES cells was confirmed by Southern blot of Spe I digested genomic DNA using a 3' external probe (black bar) which detects 8kb and 6kb bands at the wild-type and targeted locus, respectively. Homologous recombination at the 5' end was confirmed in these ES cell clones by Southern blot and both ends were reconfirmed by PCR using primers external to the targeting construct.

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Primary mouse hepatocytes. Primary hepatocytes were isolated from the littermates of wild-type and *Oct1-/-* mice by the UCSF Liver Center using the standard collagenase method (18). The cells were plated in William's E medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1% bovine serum albumin, 0.1 μM dexamethasone, 2 mM L-glutamine, 1X ITS (100X Insulin-Transferrin-Selenium from Invitrogen) at a density of 1.5 x 10^5 cells/cm^2 on collagen-coated six or 12-well plates (Becton Dickenson, Bedford, MA). After attachment (2 – 3 hours), hepatocytes were maintained in the completed medium with 0.25 mg/ml Matrigel (BD Sciences, San Jose, CA) for 16 hours followed by regular medium change and drug treatment as described below.

Drug uptake in cells. Clone 9 cells and 3T3-L1 cells were grown on regular plastic 12 and 24-well plates respectively, and HEK 293 cells were grown on poly-D-lysine coated 24-well plates to at least 90% confluence. Primary hepatocytes were plated on collagen-coated 12-well plates at a density of 1.5 x 10^5 cells/cm^2. The cells were washed once with room temperature PBS, and then incubated in the uptake buffer (MPP^+, 1-methy-4-phenylpyridinium, in phosphate-buffered saline, PBS; or metformin in serum- and antibiotic-free culture media) containing 1 μM MPP^+ [0.1 μM ^3H-MPP^+ (72 mCi/mmol, Perkin Elmer, Boston, MA) and 0.9 μM MPP^+ (Sigma, St. Louis, MO)] or various concentrations of metformin [35 μM ^{14}C-metformin (26 mCi/mmol, Moravek Biochemicals, Brea, CA) and unlabeled metformin (Sigma)]. The uptake was performed at room temperature for 2 min (MPP^+) or 10 min (metformin) and then the cells were

washed three times with ice-cold PBS. The cells were lysed with 0.1 N NaOH and 0.1% SDS and the lysate was used for scintillation counting (Beckman Instruments, Palo Alto, CA) and for the BCA protein assay (Pierce, Rockford, IL).

Kinetic studies were performed in cells stably expressing reference OCT1 and four polymorphisms. These four variants were selected for kinetic studies because they exhibited a reduced uptake of metformin and our goal was to identify the kinetic mechanisms responsible for the reduced uptake. Further, the variants had sufficiently high activities of metformin uptake to obtain accurate kinetic parameters. To obtain the K_m and V_{max} in the kinetic studies, the data were fit to the Michaelis-Menten equation: $V = V_{max} \cdot S/(K_m+S)$, where V_{max} is the maximum transport rate, K_m is the Michaelis-Menten constant at which the transport rate (V) is one half the V_{max}, and C is the concentration of substrate. GraphPad Prism (Version 4.03, GraphPad Software, Inc.) was used to fit the data.

Glucose uptake in cells. Clone 9 cells and 3T3-L1 cells were grown on 12-well plates to confluence. Two days after confluence, differentiation was initiated in 3T3-L1 cells by adding a medium containing 10 μg/ml insulin, 0.5 mM 1-methyl-3-isobutylxanthine (IMBX), and 1 uM dexamethasone (19). After 48 hours, the medium was switched back to regular growth medium with 10 μg/ml insulin. The cells were considered satisfactory for studies when at least 90% were differentiated as adipocytes. Twenty-four hour before initiation of the glucose uptake experiments, the medium was changed to serum-free DMEM. The cells were incubated in the medium containing 5-aminoimidazole-4-carboxamine-1-β-D-ribofuranoside (AICAR) or metformin for 2 hours before the initiation of glucose uptake. The uptake substrate consisted of 3-O-methyl-D-

[1-^{3}H]glucose (^{3}H-3-OMG, 4.0 Ci/mmol, GE Healthcare Bio-Sciences, Piscataway, NJ) plus unlabeled 3-OMG (Sigma). The rate of cytochalasin B-inhibitable 3-OMG uptake was measured as described previously with minor modifications (20). After the uptake was terminated by removal of the medium, cells were washed three times with ice-cold PBS containing 0.1 mM phloretin. As described above, the cells were then lysed and the lysate was used for scintillation counting and protein determination.

Lipid accumulation in 3T3-L1 cells. The 3T3-L1 cells were grown on 6-well plates, and were differentiated as described above. AICAR and metformin were added to the medium before, 3 days after, or 5 days after the initiation of differentiation. The intracytoplasmic lipids of differentiated 3T3-L1 cells were stained with Oil red O, and the dye was extracted with isopropyl alcohol and quantitated spectrophotometrically, as previously described (19).

Hepatocyte glucose production. Glucose production in primary mouse hepatocytes was measured by modifying a previously described method (4). Hepatocytes on collagen-coated 6-well plates (1.5 x 10^5 cell/cm^2) were incubated in a medium containing Matrigel for 16 hours and then were maintained in the regular medium overnight before the glucose production experiments. For the glucose production experiments, the hepatocytes were incubated in bicarbonate-buffered saline medium containing 10 mM L-lactate, 1 mM pyruvate, 0.3 μM glucagon (Sigma), with or without metformin (1 mM). The glucose concentration in the medium was measured with the glucose oxidase kit from Sigma.

Animal experiments. Age-matched *Oct1+/+* and *Oct1-/-* mice were used for all animal experiments. For measuring tissue distribution of metformin, male mice were fasted for 16 hours, then given an oral gavage dose of 15 mg/kg metformin in saline with 0.2 μCi/g of ^{14}C-metformin, and sacrificed one hour later. The liver, kidney, heart, spleen, intestine, brain, and femoral muscle were removed immediately. All the tissues were weighed and homogenized with PBS. For the metformin pharmacokinetic study, mice were fasted for 12 hours, then given an oral gavage dose of 15 mg/kg metformin in saline with 0.2 μCi/g of ^{14}C-metformin, and placed in metabolic cages for 24 hours. The food was re-administered four hours after metformin treatment. Blood samples were collected at specific time points by tail bleeding into heparinized micro-hematocrit capillary tubes (Fisher, Pittsburgh, PA). Urine and feces were collected from tubes attached to the cages. Plasma was isolated by centrifuging the capillary tubes in a microhematocrit centrifuge (Thermo Electron Corporation, MA). Metformin from tissue and fecal homogenates, plasma, and urine were measured by scintillation counting. The pharmacokinetic parameters were obtained by fitting the raw data using a non-compartmental model with WinNonlin (Pharsight, Mountain View, CA).

To study the *in vivo* pharmacologic effects of metformin, six-week old mice were fed high-fat diets (Diet D12492, Research Diets, New Brunswick, NJ) for eight weeks. Mice were then injected intraperitoneally with 0.9% sterile saline or metformin in 0.9% sterile saline in a manner similar to that described by Shaw *et al.* (7). Instead of 250 mg/kg metformin for 3 days, we treated the mice with 50 mg/kg metformin, a dosage approximating maximal doses used to treat diabetic patients, for 5 days. In brief, we

collected blood at 12:00 PM (day 0) by tail bleeding from the mice fasted for 18 hours. Food was then resumed and the mice were injected with metformin or saline at 10:00AM for the next five days (day 1 – day 5). At 6:00PM on day 4, the mice were again fasted. Blood was collected by tail bleeding at 12:00PM on day 5. After blood collection, the animals were sacrificed and livers rapidly removed by freeze clamping. Plasma was isolated as described above, and glucose concentration was measured using the glucose oxidase assay kit (Sigma).

Clinical study. The study protocol was reviewed and approved by Committee on Human Research at UCSF. The subjects were selected from the participants recruited for a large project named SOPHIE (Study Of PHarmacogenetics In Ethnically Diverse Populations). At the time of initial enrollment, SOPHIE participants are consented to be re-contacted about their willingness to participate in subsequent clinical pharmacogenetic research studies. Previously we had identified the ethnic-specific allele frequencies of common variants of *OCT1* with reduced or no function (13). The current clinical study was initially based on these data and designed to assess the effects of OCT1-R61C, G401S and G465R on metformin response. Because these three variants mainly occur in individuals with European ancestry, our initial recruitment was limited to healthy male or female European Americans from SOPHIE only with OCT1 reference alleles, or with one of these three variants. However, during the cellular study, we identified another common OCT1 variant (420Del) with reduced metformin but not MPP^+ uptake (Figure 3.13A). All participants were then genotyped for this polymorphism too. The characteristics and genotypes for the subjects are summarized in Table 3.1.

Table 3.1. The characteristics and OCT1 polymorphisms of human healthy volunteers in the clinical study.

Subject No.	Polymorphism[a]	Body Weight (kg)	Height (cm)	Age (year)	Gender[d]
1	Reference	82.1	186	28	M
3	Reference	58.7	169	36	F
4	Reference	67	175	25	M
7	Reference	64.7	162	40	F
9	Reference	82.2	185	36	M
10	Reference	76.6	179	31	M
12	Reference	68.2	177	27	F
16	Reference	52.2	171	27	F
Subtotal		68.9 ± 10.8	176 ± 8.1	31.3 ± 5.4	4M, 4F
2	R61C	102	188	35	M
6	G401S	80	184	34	M
8	R61C[b]	97.1	186.3	27	M
11	R61C	60.9	163	34	F
13	420Del	67.9	173	35	F
14	G465R,420Del	66.3	160	26	F
15	G465R,420Del	74.2	159	26	F
17	R61C	75.1	186	27	M
18	G174S[c],420Del	79.1	168	40	F
19	G465R,420Del	62.2	162	32	F
20	G401S	69	183	25	M
21	G401S	55.4	157	19	F
Subtotal		74.1 ± 14.0	173 ± 12.3	30.0 ± 5.9	6M, 6F

[a] Individuals who carried any of the four polymorphisms, OCT1-R61C, OCT1-G401S, OCT1-420del and OCT1-G465R, are referred to as individuals with an OCT1-variant, and those who had the reference allele at all four positions are termed individuals with OCT1-reference alleles. Except subject 8, all other subjects were heterozygotes for the polymorphisms studied.

[b] Homozygote for R61C.

[c] Found in a volunteer after re-sequencing, and not determined on the cellular phenotype of OCT1 function (MPP$^+$ and metformin uptake).

[d] M: male; F: female

After informed consent was obtained, healthy individuals with known *OCT1* genotypes were recruited into this open label study. Once enrolled, participants were advised to maintain stable activity levels (without periods of strenuous exercise) for seven days before the formal study. Approximately seven days prior to study, subjects met with a dietitian to create a three-day meal plan which maintained carbohydrate intake at 200-250 g/day. The volunteers recorded their food intake in a three-day food diary. Consistent and adequate carbohydrate intake would reduce variation in the results of the oral glucose tolerance test (OGTT), our primary determinant of metformin action in this study. After the initial three-day diet-maintained period, subjects were admitted (day 0) to the General Clinical Research Center (GCRC) at San Francisco General Hospital (SFGH), and remained at this center for the duration of the study. On the following morning (day 1), a three-hour oral glucose tolerance test (OGTT, 75 g glucose) was conducted. Subjects were then dosed with 1000 mg metformin in the evening followed by a dose of 850 mg on the morning of day two two-hours before a second OGTT. The plasma glucose concentrations from OGTT before and after metformin treatment were compared between individuals who carry the decreased or non-functional OCT1 variants and those who carry the reference alleles.

Immunoblots. Cultured cells were lysed at 4°C for 20 minutes in buffer containing 20 mM Tris pH 7.4, 1% Triton-X 100, 150 mM NaCl, 250 mM sucrose, 50 mM NaF, 2.5 mM $Na_3P_2O_4$, 2 mM DTT and 10 mM Na_3VO_4 with the protease inhibitors dissolved from Complete® protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN). Liver samples were homogenized in ice-cold lysis buffer using a tissue

homogenizer at 4°C for 20 minutes. After centrifugation for 20 minutes at 14,000 g at 4°C, the supernatants were removed for determination of protein content and separated on 10% SDS-PAGE gels. Proteins (40 μg) from the supernatant were separated and transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C with Tris-buffered saline with 0.05% Tween 20 and 5% nonfat milk. Immunblotting was performed following standard procedures and the signals were detected by chemiluminescence reagents (Amersham). Primary antibodies were directed against AMPKα, AMPKα phosphorylated at Thr172, ACC phosphorylated at Ser79, and β-actin (all from Cell Signaling Technology, Danvers, MA).

RNA isolation and RT-PCR. Three Octs (Oct1, Oct2, and Oct3) have been cloned. Metformin is a substrate for Oct1 and Oct2 (10, 11, 21) but not for Oct3 (unpublished data from this laboratory). mRNA transcripts of Oct1 and Oct2 in different cell lines were detected by RT-PCR. Cells were cultured in 6-well plates as described above. Total RNA was extracted using Trizol (Invitrogen). To detect Oct1, we used one-step RT-PCR kit (Roche Applied Science). Thirty-five amplification cycles were used in the PCR. To detect Oct2, the first-strand cDNA was synthesized from 1 μg of total RNA using SuperScript™ III First-Strand Synthesis System kit (Invitrogen) and the resulting cDNAs were used for PCR with 40 amplification cycles. The mouse, rat, and human GAPDHs were used as the expression control for Clone 9 cells, 3T3-L1 cells, and HEK293 cells, respectively. Primers for PCR are provided in Table 3.2 Real-time PCR was also done to quantify the transcript levels of human OCT1 or its variants in stable

Table 3.2. Primers used in RT-PCR to detect Oct1, Oct2 and GAPDH.

Gene	Species	Primers (5'→ 3')
Oct1	Mouse	Sense: TTGGAGAGTTTGGCTGGTTC
		Anti-sense: CACCAGGAGGCAGAGCTTAC
	Rat	Sense: TTGGAGAGTTTGGCTGGTTC
		Anti-sense: CACCAAGAGACAGAGCTTAC
	Human	Sense: CTGTGTAGACCCCCTGGCTA
		Anti-sense: GTGTAGCCAGCCATCCAG
Oct2	Mouse	Sense: AACCCTTCGTTCCTGGACTT
		Anti-sense: GTTGACCAGGCAGACCATTT
	Rat	Sense: CTCAGCCAGTGCATGAGGTA
		Anti-sense: AAAGCGAAACACCAACATCC
	Human	Sense: CCTGGTATGTGCCAACTCCT
		Anti-sense: CACCAGGAGCCCAACTGTAT
GAPDH	Mouse	Sense: GGGTGTGAACCACGAGAAATATG
		Anti-sense: GAAGGCCATGCCAGTGAGC
	Rat	Sense: GGGTGTGAACCACGAGAAATATG
		Anti-sense: GAAGGCCATGCCAGTGAGC
	Human	Sense: AATCCCATCACCATCTTCCA
		Anti-sense: TGTGGTCATGAGTCCTTCCA

HEK293 cells. cDNA (20 ng) was used in quantitative real time PCR with the OCT1 probes from ABI using a ABI Prism 7700 instrument.

Statistical analysis. Unless indicated, the data are presented as mean ± standard deviation (SD), and from a representative experiment performed in triplicate or quadruplicate. Unless indicated, all experiments are repeated at least twice. One or two tailed *Student's* t-test was applied to analyze data, when appropriate, as indicated in the figure legends. For multiple comparison tests, *ANOVA* was used followed by *Dunnett's* test. A P value of < 0.05 was considered statistically significant.

Results

Oct activity is a determinant of metformin response in cell lines. To understand whether Oct1 plays a critical role in defining metformin's pharmacological effects, we first studied the effects of metfomin in cells with conserved AMPK signaling pathways but different levels of Oct activity (Figure 3.2 and 3.3). 5-Aminoimidazole-4-carboxamine-1-β-D-Ribofuranoside (AICAR), an AMPK activator, stimulated AMPK phosphorylation and glucose uptake in rat hepatocyte-derived Clone 9 cells (5). These cells retain Oct activity as demonstrated by Oct1 expression (data not shown) and the uptake of the typical substrate 1-methy-4-phenylpyridinium (MPP^+) and metformin with and without an Oct inhibitor (Figure 3.2A). In contrast, 3T3-L1 adipocyte, an established model for studying AMPK signaling pathways (22), exhibited negligible Oct expression, which was apparent only in highly differentiated 3T3-L1 cells (data not shown), and little

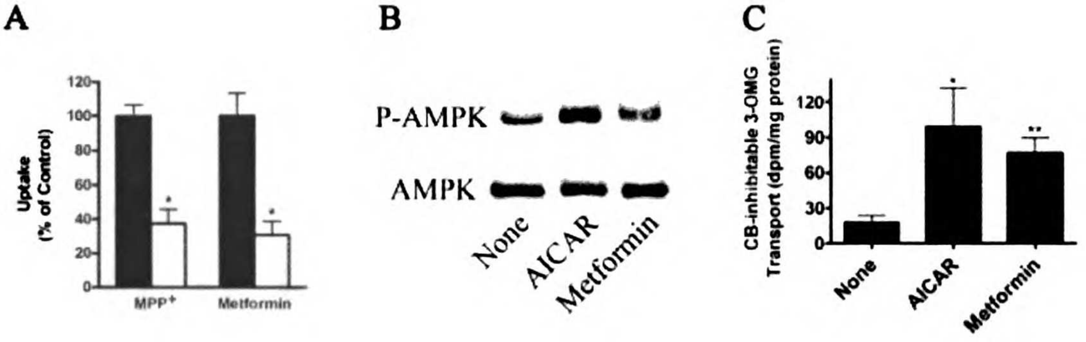

Figure 3.2. Oct activity and metformin responses in Clone 9 cells. **(A)** Clone 9 cells exhibit Oct activity, as demonstrated by significantly reduced uptakes of the typical Oct substrate MPP^+ (1 μM) and metformin (250 μM) in the presence of the Oct inhibitor, quinidine (100 μM, white bars) *versus* those without quinidine (control, black bars). The uptake times were 2 minutes for MPP^+ and 10 minutes for metformin, respectively. For uptake rates, the percentage of control (uptake in the absence of quinidine) is used to scale the two compounds in the same figure. $^*P < 0.001$ *vs.* respective controls (two-tailed *Student's t*-test). **(B)** AMPK phosphorylation at Thr172 was stimulated by both AICAR and metformin in Clone 9 cells. The AMPK activator AICAR was used as a positive control. The cells were treated with metformin (2 mM) or AICAR (2 mM) for 4.5 hours. Cell extracts were detected with polyclonal antibodies against phospho-AMPKα (Thr172) (top) and AMPKα (bottom) respectively. **(C)** Both metformin and AICAR increased 3-OMG transport in Clone 9 cells. The cells were treated with metformin (2 mM) or AICAR (2 mM) for two hours before initiation of 3-OMG transport. 3-OMG transport was measured as described in the Methods section. $^*P < 0.01$, $^{**}P < 0.05$ *vs.* none (*ANOVA* and *Dunnett's* test).

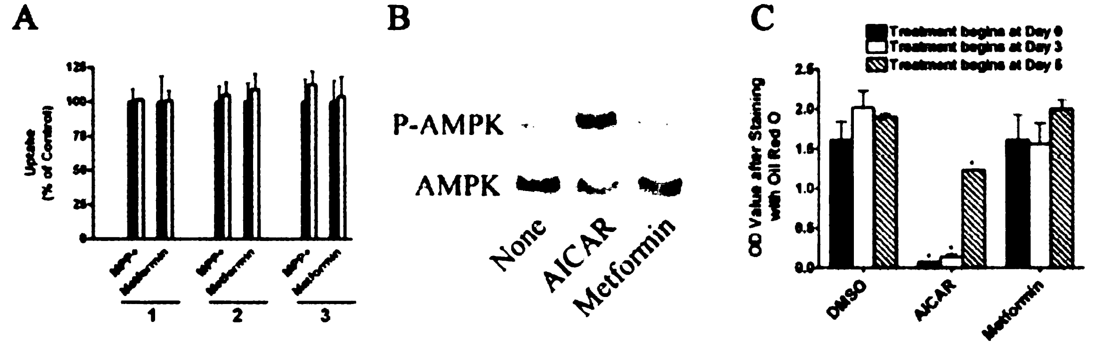

Figure 3.3. No Oct activity and metformin responses in 3T3-L1 cells. **(A)** No Oct activity was detected in 3T3-L1 cells of various differential stages. Uptake studies were done as described in Figure 3.2A. 3T3-L1 cells were differentiated as described in the Methods section. The cells for uptake experiments are 1: pre-adipocytes; 2: 3T3-L1 differentiated for 5 days; and 3: 3T3-L1 differentiated for 10 days. **(B)** Metformin (2 mM) had little effect on AMPK phosphorylation in 3T3-L1 cells. **(C)** Metformin (2 mM) treatment during differentiation did not affect lipid accumulation in 3T3-L1 cells, in contrast to the significant effects by AICAR (2 mM). The cellular lipids were stained with Oil Red O, and lipid content was determined by measuring OD of the dye extracted with isopropyl alcohol. *$P < 0.01$ *vs.* 0.05% DMSO, two-tailed *Student's* t-test.

Oct-mediated uptake (Figure 3.3A). Although the cell-permeable AICAR stimulated AMPK phosphorylation and glucose uptake in both cell lines (5, 22), metformin caused markedly different responses. Metformin stimulated AMPK phosphorylation and glucose uptake in Clone 9 but not in 3T3-L1 cells (Figures 3.2B, 3.2C, 3.3B, and data not shown). Furthermore, whereas AICAR inhibited lipid accumulation during 3T3-L1 differentiation as assessed by Oil Red O staining (23), no such effect was observed for metformin (Figure 3.3C).

Since differences in metformin responses between Clone 9 and 3T3-L1 cells may have been due to differences between the cell types unrelated to Oct activity, we compared metformin-stimulated AMPK activation in cells transfected with Oct1 *versus* control (empty vector transfected) cells. The accumulation of metformin was time-dependent and substantially increased in cells stably transfected with OCT1 (HEK-OCT1) (Figures 3.4A, B). AMPK activation by metformin was significantly greater in HEK-OCT1 than in empty vector transfected cells (Figure 3.5). When cells were exposed for 1 hour to metformin (250 μM), AMPK activation was apparent in HEK-OCT1 cells with little activation in control cells (Figure 3.4C). The data support the hypothesis that Oct1 modulates metformin-stimulated AMPK phosphorylation.

***Oct1* deletion results in reduced metformin uptake and response in primary mouse hepatocytes.** In an effort to understand the role of Oct1 in metformin action in the liver, a key target for metformin (1), we used gene targeting to construct an *Oct1* knockout (*Oct1-/-*) mouse (Figure 3.1). Consistent with the study of Jonker *et al.* (24), our *Oct1-/-* mice appeared to be healthy. As expected, metformin uptake in primary

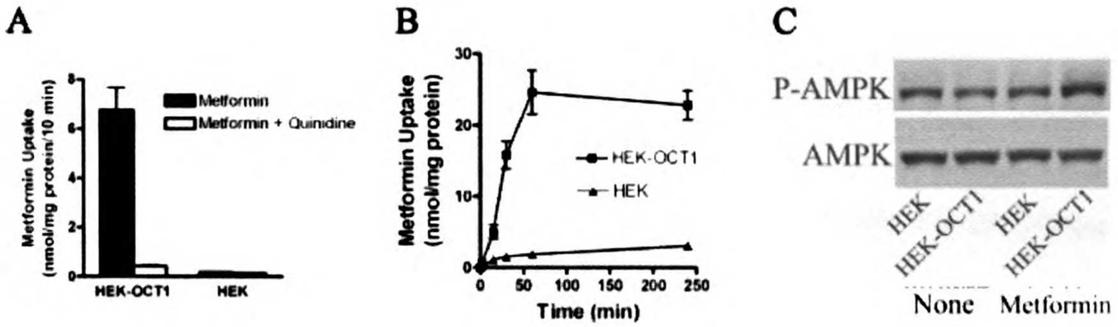

Figure 3.4. Overexpressing human OCT1 in HEK-293 cells increases metformin uptake and metformin-stimulated AMPK phosphorylation. **(A)** The uptake of metformin in HEK-293 cells was markedly increased by stable overexpression of human OCT1 in the cells. The uptake experiments were performed as described in the Methods and in Figure 3.2A. HEK represents empty vector transfected cells. **(B)** The uptake of metformin in the HEK-293 cells overexpressing human OCT1 was time-dependent. **(C)** AMPK phosphorylation by metformin in HEK-293 cells was markedly increased by stably overexpressing human OCT1 in the cells. The cells were treated with metformin (250 μM) for one hour. Cell extracts were detected with polyclonal antibodies against phospho-AMPKα (Thr172) (top) and AMPKα (bottom) respectively.

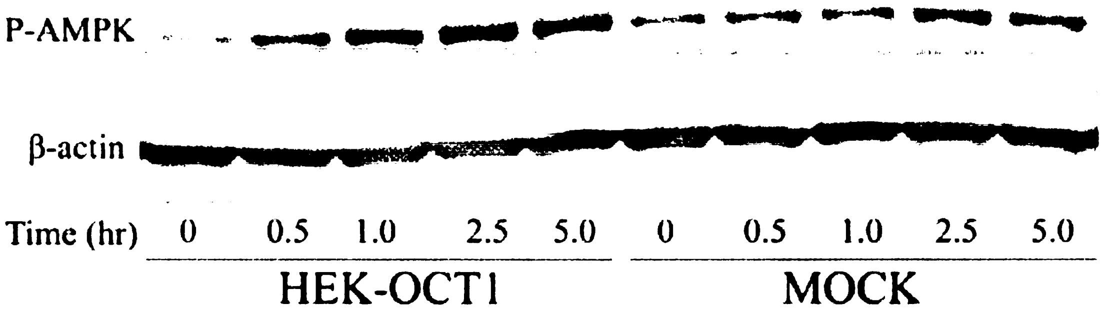

Figure 3.5. Time-dependent AMPK phosphorylation in HEK-OCT1 cells and mock cells. The cells were treated with metformin (250 μM) for 0 – 5.0 hours. Cell extracts were detected with polyclonal antibodies against phospho-AMPKα (Thr172) (top) and β-actin (bottom) respectively.

mouse hepatocytes was significantly lower (3.4-fold, $P < 0.0001$) in *Oct1-/-* cells compared to that in cells from even heterozygous mice (Figure 3.6A). Importantly, phosphorylation of AMPK and acetyl-CoA carboxylase (ACC), an AMPK target, by metformin was substantially reduced in *Oct1-/-* hepatocytes in comparison to those in wild-type hepatocytes (Figure 3.6B). Quinidine, an Oct inhibitor, decreased the phosphorylation of AMPK and ACC by metformin in wild-type hepatocytes (Figure 3.7). Downstream, metformin-stimulated AMPK phosphorylation results in a decrease in hepatic gluconeogenesis (4, 7). Metformin (1 mM) significantly suppressed glucagon-stimulated glucose production in hepatocytes from wild-type mice (30% suppression, $P < 0.001$) but not in hepatocytes from *Oct1-/-* mice (Figure 3.8). Collectively, these data are consistent with those from the cell lines and suggest that the effect of metformin on AMPK and ACC in the hepatocyte is modulated by Oct1. Thus, Oct1 appears to play a key role in determining one of the major pharmacologic effects of metformin, inhibition of hepatic gluconeogenesis.

***Oct1* deletion results in reduced hepatic accumulation and therapeutic response of metformin in mice.** Next, we examined the role of Oct1 in metformin disposition and glucose-lowering effects *in vivo*. Similar to the previous report of metformin pharmacokinetics following intravenous doses (10, 11), we observed that following a single oral dose (15 mg/kg), the plasma concentrations of metformin were similar in *Oct1-/-* and wild-type mice (Figure 3.9A) but that the hepatic accumulation was significantly greater in wild-type mice (4.2-fold, $P < 0.001$; 1 hour after dosing) than in *Oct1-/-* mice (Figure 3.9B). No accumulation difference was measured in other major

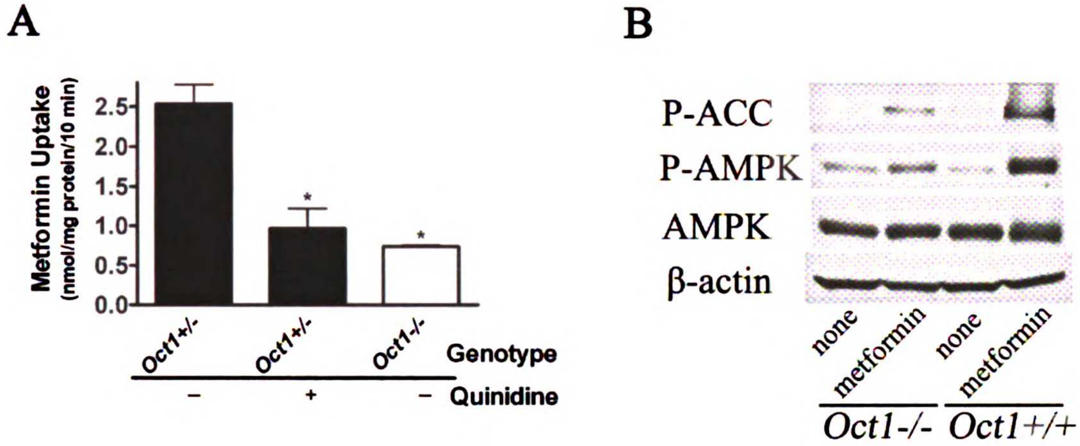

Figure 3.6. Oct1 deletion results in reduced metformin uptake and response in primary hepatocytes from mouse. **(A)** Metformin uptake was less in the primary hepatocytes isolated from *Oct1* knock-out (*Oct1-/-)* mice than in those with a normal *Oct1* allele (*Oct1+/-*). The uptake of metformin (250 μM) was performed for ten minutes in the presence or absence of 100 μM quinidine, where indicated. *$P < 0.01$ *vs Oct1+/-* without quinidine (*ANOVA* and *Dunnett's* test). **(B)** Metformin resulted in less phosphorylation of AMPK and ACC in *Oct1-/-* hepatocytes than in *Oct1+/+* hepatocytes. The cellular extracts from primary hepatocytes treated with or without metformin (250 μM) for four and half hours were detected with polyclonal antibodies against phospho-ACC (Ser 75), phospho-AMPKα (Thr172), AMPKα, and β-actin respectively.

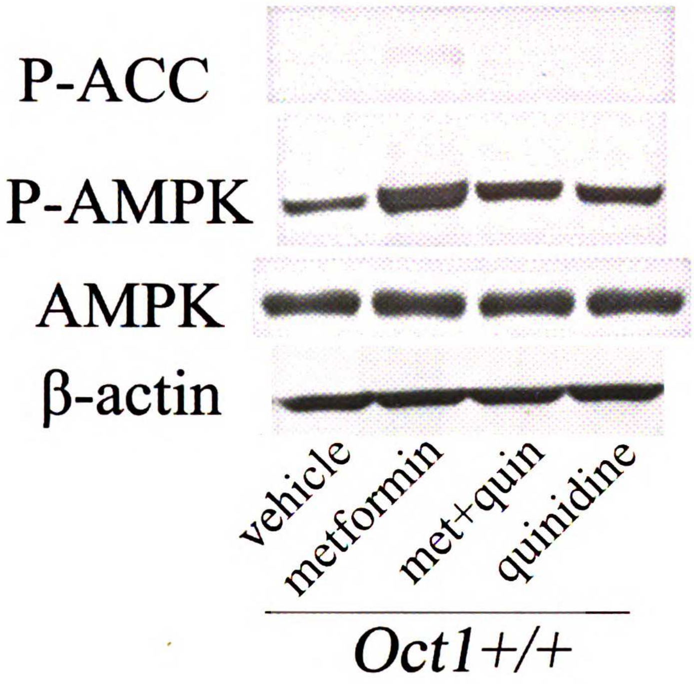

Figure 3.7. Treatment with the Oct inhibitor quinidine reduced the stimulation of AMPK phosphorylation and thus ACC phosphorylation by metformin in *Oct1+/+* hepatocytes. Where indicated, 100 μM quinidine was added 30 minutes before metformin (250 μM) treatment.

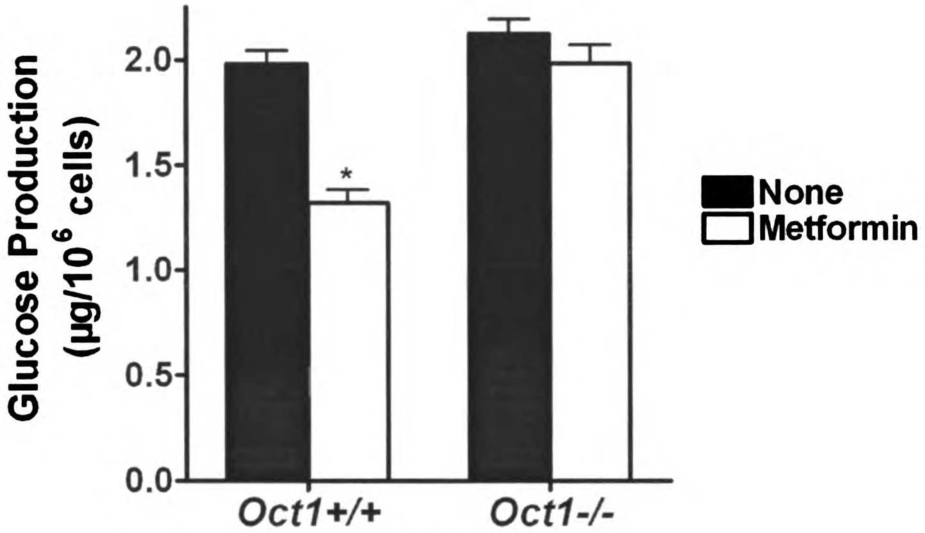

Figure 3.8. Metformin suppressed glucagon-stimulated glucose production in *Oct1+/+* hepatocytes with no effect in *Oct1-/-* hepatocytes. Metformin (1 mM) was added 2 hours before glucose measurement. The primary hepatocytes were isolated and cultured as described in the Methods. *$P < 0.001$ *vs* untreated, two-tailed *Student's* test.

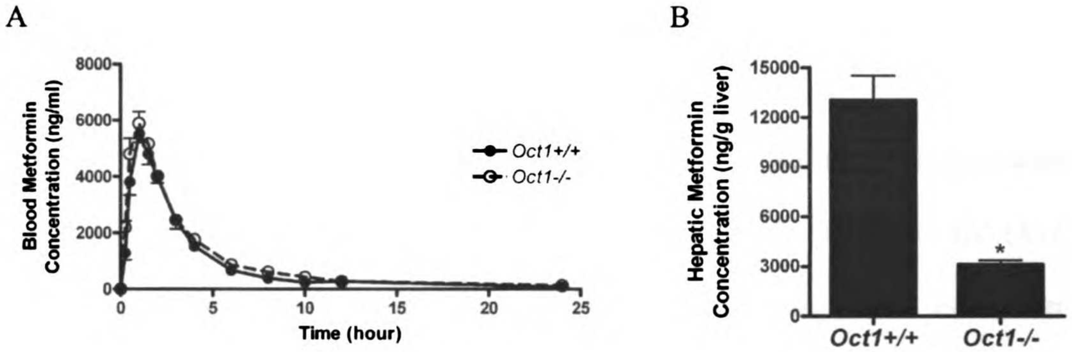

Figure 3.9. *Oct1* deletion results in reduced hepatic metformin accumulation in mice orally dosed with metformin. **(A)** The systemic pharmacokinetics of metformin was similar between *Oct1+/+* mice and *Oct1-/-* mice after an oral dose. Shown here are blood metformin concentration-time profiles. The mice (n = 4 per group) were given an oral dose of metformin (15 mg/kg containing 0.2 mci/kg of ^{14}C-metformin), approximating the single dose of 1000 mg in humans. The radioactivity in blood was determined and converted to mass amounts. Data represent mean ± SD. **(B)** Hepatic metformin accumulation after an oral dose was much higher for *Oct1+/+* mice than for *Oct1-/-* mice. The mice (n = 4 per group) were sacrificed one hour after the oral dose and the livers were removed immediately. The radioactivity determined in liver homogenates was converted to mass amounts. Data represent mean ± SD. *$P < 0.001$ *vs Oct1+/+*, two tailed *Student's* t-test.

organs (Figure 3.10). Importantly, after metformin treatment, phosphorylation of both AMPK and ACC was substantially reduced in livers from *Oct1-/-* mice compared to those from wild type mice (Figure 3.11).

The primary end-therapeutic effect of metformin in the treatment of diabetes is to lower blood glucose levels. To increase fasting blood glucose levels, we fed *Oct1-/-* and *Oct1+/+* mice a high fat diet for eight weeks. Then mice were treated with either saline or metformin for five days. Although no differences were observed in baseline fasting blood glucose levels between *Oct1-/-* and *Oct1+/+* mice high fat diets, we observed that metformin significantly reduced fasting plasma glucose levels by more than 30% in wild-type mice fed the high fat diet ($P = 0.012$), but not in the *Oct1-/-* mice on the same diet (Figure 3.12). These data suggest that *in vivo* Oct1 serves a critical function in metformin's primary therapeutic effect of lowering fasting plasma glucose levels.

***OCT1* polymorphisms modulate metformin uptake and response in cells.** Previously, we and others showed that human *OCT1* is a highly polymorphic gene (13-15). To determine whether *OCT1* polymorphisms modulate metformin response, we measured the uptake of metformin in stable cell lines expressing empty vector, OCT1-reference, and twelve OCT1 nonsynonymous variants (Figure 3.13A). Compared to OCT1-reference, seven OCT1 variants exhibited significantly reduced metformin uptake, despite similar levels of mRNA (Figure 3.14). Kinetic studies in cells expressing four of the reduced-function variants indicated that the decrease in uptake was probably due to reduced V_{max} (Figure 3.13B, Table 3.3). Notably, phosphorylation of AMPK and ACC by metformin was reduced in cells expressing the non-functional or reduced function

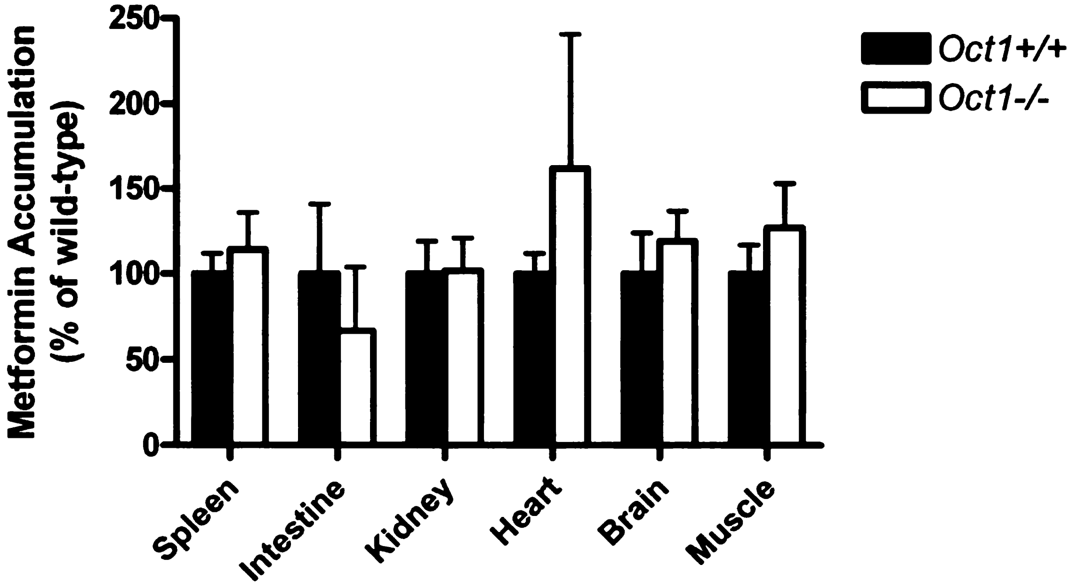

Figure 3.10. Metformin accumulation in different tissues of *Oct1+/+* and *Oct1-/-* mice. The mice (n = 4 per group) were sacrificed one hour after the oral dose (15 mg/kg), and the tissues were removed immediately. The radioactivity in tissue homogenates was counted and converted to mass amount. Data represent mean ± SD. Liver is not shown here (See Figure 3.9B).

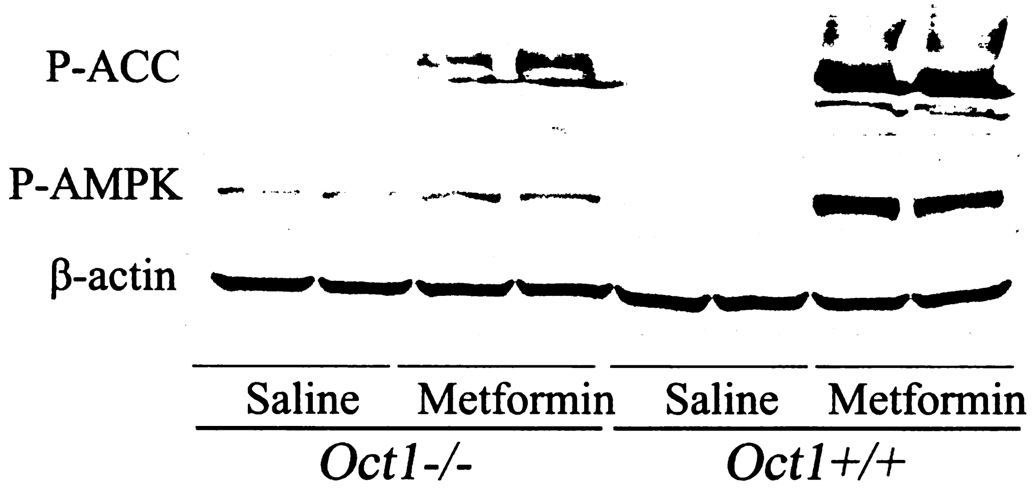

Figure 3.11. Oct1 was required for metformin to fully stimulate hepatic AMPK phosphorylation and ACC phosphorylation in mice. A daily dose of metformin, 50 mg/kg, or saline were administered IP for 3 consecutive days to 10-week old male mice. The mice were sacrificed 1 h after the IP administration on the third day. Liver extracts were detected with polyclonal antibodies against phospho-ACC (Ser 75), phospho-AMPKα (Thr172), and β-actin respectively.

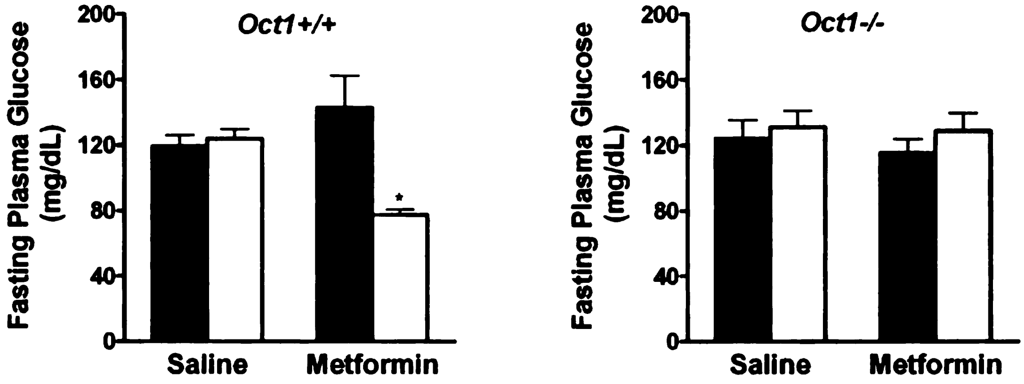

Figure 3.12. Oct1 is required for metformin to lower fasting plasma glucose in mice. The six-week old *Oct1+/+* mice and *Oct1-/-* mice (n = 5 - 8 per group) were administered a high-fat diet for eight weeks, and 18-hour fasting plasma glucose concentrations were measured before (black bars) and after (white bars) 5-day intraperitoneal saline or metformin (50 mg/kg each day). Data represent mean ± SD. **P* = 0.012 *vs* day 0, two tailed *Student's* t-test.

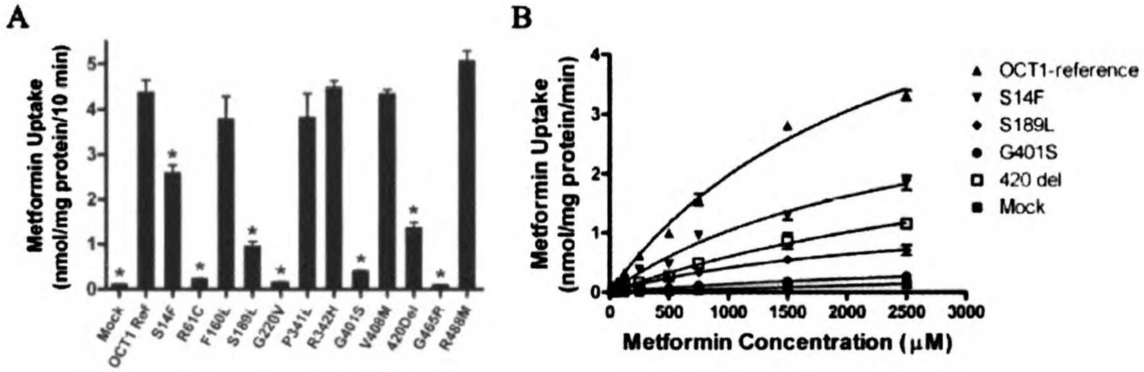

Figure 3.13. OCT1 genetic variants are associated with different accumulation rates of metformin in stably transfected HEK293 cells. **(A)** Uptake of ^{14}C-metformin by cell lines stably expressing human OCT1 and its variants. Cells expressing OCT1 and its variants were incubated with ^{14}C-metformin (250 μM) for 10 minutes. Seven OCT1 variants exhibited reduced metformin uptake as compared to OCT1-reference. Data are expressed as mean ± SD for samples analyzed in quadruple. $^{*}P < 0.001$ compared with the reference, two tailed *Student's* t-test. **(B)** Metformin kinetics in cell lines expressing reduced function variants of OCT1. Four of the reduced function variants shown in A had enough activity to perform kinetic studies with metformin. The data from metformin uptake at eight different concentrations are plotted. The variants had significantly different V_{max} with a similar K_m (Table 3.3).

Table 3.3. Kinetic parameters of metformin uptake in HEK-293 cells stably transfected with human OCT1 or OCT1 variant. The K_m and V_{max} values determined from the fit of the data in Figure. 3.13B are shown here. Although the K_m values are similar, there is a significant change in the V_{max} values between the variants. Values are mean ± SE.

	Reference	S14F	S189L	G401S	420 del
Vmax (nmol/min/mg protein)	6.74 ± 0.88	3.77 ± 0.65	1.40 ± 0.05	0.67 ± 0.88	3.31 ± 0.56
Km (mM)	2.42 ± 0.52	2.65 ± 0.74	2.41 ± 0.15	3.88 ± 0.75	4.56 ± 1.08

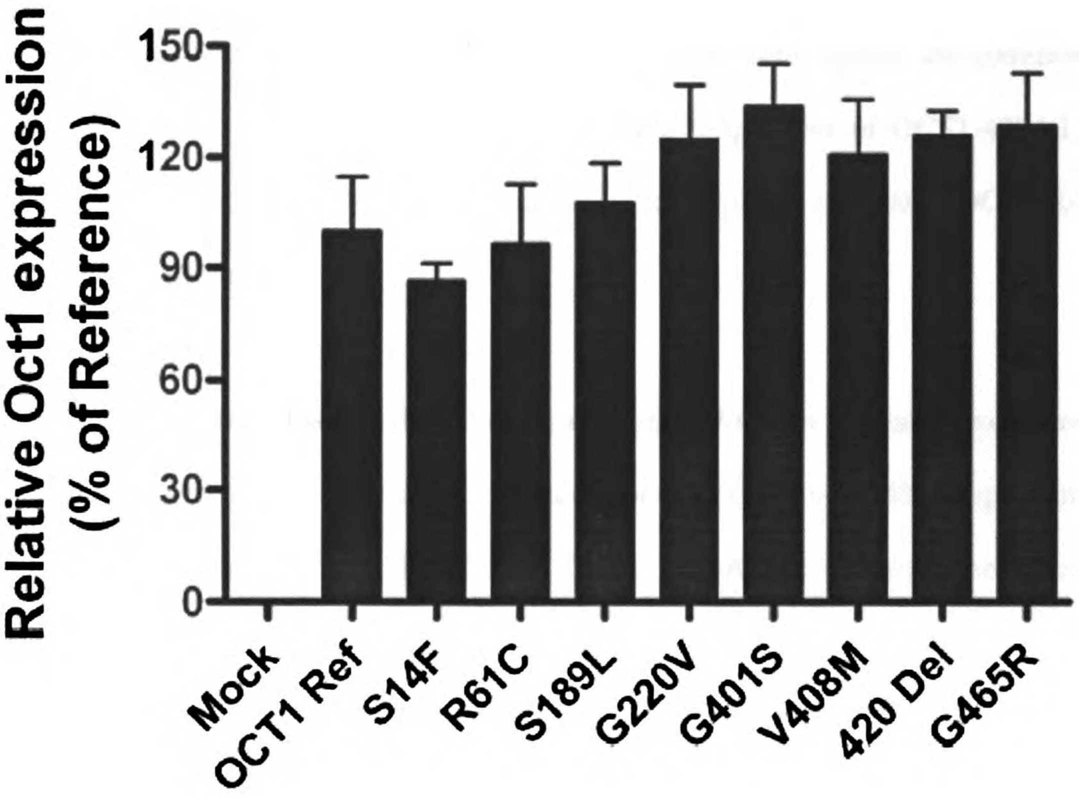

Figure 3.14. *OCT1* transcript levels in cell lines stably expressing human OCT1 and its variants as determined by RT-PCR. The total RNA amount of OCT1 reference and all the variants was similar with a remarkable increase over mock transfected cells. Data are expressed as mean ± SD for samples analyzed in triplicate with the level in OCT1-reference transfected cells set at 100%.

variants compared to those expressing OCT1-reference and OCT1-V408M, a variant with normal metformin uptake (Figure 3.15), although reduced AMPK phosphorylation did not consistently correspond to reduced uptake of metformin (see Discussion). These data in cells suggest that OCT1 polymorphisms modulate responses to metformin. Of significance, polymorphisms of OCT1 with reduced metformin uptake are common in human populations (13-15). For example, the allele frequencies of OCT1-420del are 19% and 5% in Caucasians and African Americans, respectively, and that of OCT1-R61C is 7.2% in Caucasians (13).

***OCT1* polymorphisms affect the response to metformin in healthy volunteers.** To extend the cellular studies further, we conducted a genotype to phenotype clinical study in healthy volunteers with different OCT1 genotypes. Although the glucose-lowering effect of metformin is not apparent in non-diabetic subjects (25), this effect can be detected in healthy subjects after plasma glucose levels are increased by administering oral glucose or conducting an "oral glucose tolerance test (OGTT)"(26). We observed similar plasma glucose levels and areas under the glucose concentration-time curves (AUC) after OGTT between volunteers carrying only reference *OCT1* alleles and those carrying a reduced function variant of OCT1 (AUC: 19800 ± 1480 *versus* 19800 ± 2520 min·mg/dL, $P = 0.955$; Figure 3.16A). However, after metformin treatment, volunteers carrying OCT1 variants had significantly higher plasma glucose levels for most of the sampling time points during the 180-min OGTT than those carrying only reference *OCT1* alleles (Figure 3.16B); and thus glucose AUC was significantly greater for the volunteers carrying the OCT1 variants as compared to those carrying only reference alleles (18200 ±

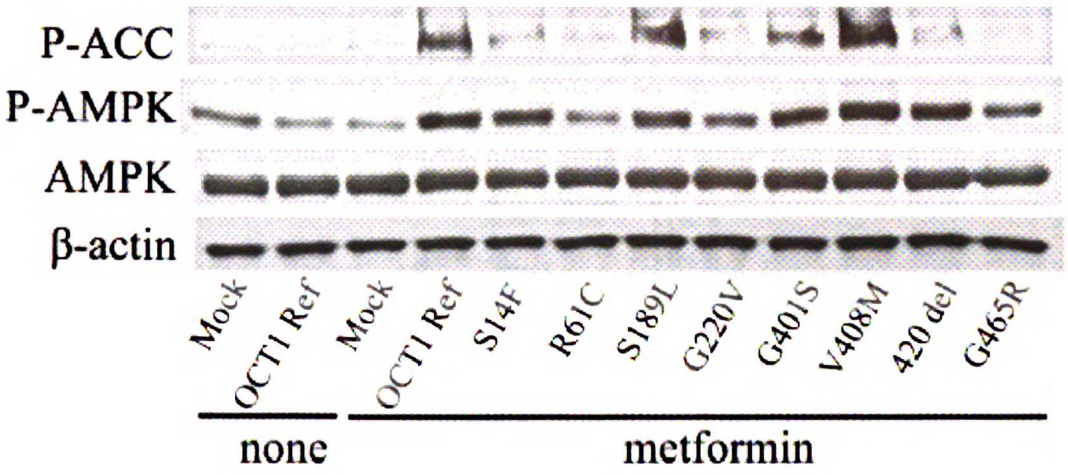

Figure 3.15. Metformin-stimulated AMPK phosphorylation and ACC phosphorylation in cell lines stably overexpressing human OCT1 and its variants. Immunoblots were performed against phospho-ACC (Ser 75), phospho-AMPKα (Thr172), AMPKα, and β-actin respectively following incubation with metformin (250 μM).

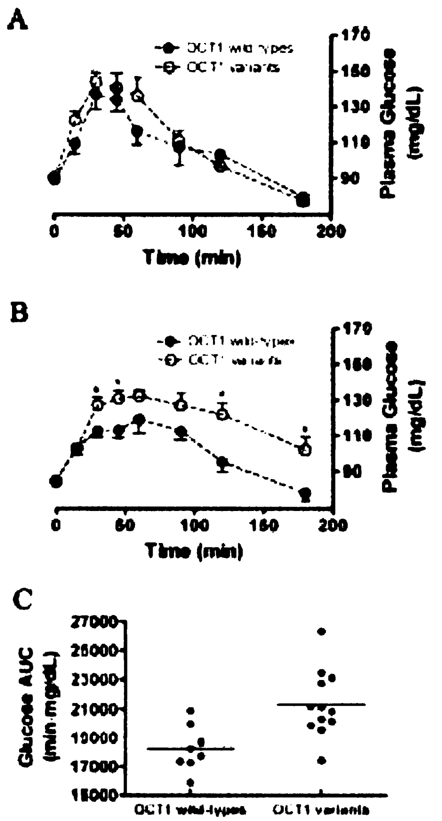

Figure 3.16. OCT1 genetic variants are associated with different responses to metformin in healthy human volunteers. **(A)** The time course of plasma glucose concentrations for a baseline oral glucose tolerance test (OGTT) without metformin treatment in healthy subjects having reference *OCT1* alleles (n = 8) and those having at least one reduced-function *OCT1* allele (n = 12). The data are expressed as mean ± SE. **(B)** The time course of plasma glucose concentrations for OGTT after metformin treatment in the same healthy subjects studied in A. The data are expressed as mean ± SE; $^*P < 0.05$ compared with volunteers with only reference *OCT1* alleles, unpaired *Student's* t-test. **(C)** The glucose exposure with OGTT (the area under the time-plasma glucose concentration curve, AUC) after metformin treatment for healthy subjects studied in B. The lines in the figure represent mean values for the two groups. The mean value for volunteers with reference *OCT1* alleles only is significantly lower than that for the group with the variants. $P = 0.004$, unpaired *Student's* t-test.

1600 *versus* 21300 ± 2290 min·mg/dL, *P* = 0.004; Figure 3.16C). When we assessed differences in glucose AUC with and without metformin for each individual and compared the differences between individuals with reference alleles and those with variant alleles, we also observed a significant difference (*P* = 0.001). These results are consistent with the data from the mice further supporting our finding that Oct1 is critical for the therapeutic response of metformin and suggesting that genetic variation in OCT1 may cause variation in response to metformin. A more pronounced effect of OCT1 genotypes on metformin therapy may be expected among diabetic patients.

Discussion

In this study, we performed a comprehensive analysis of the role of Oct1 in response to metformin. We began our studies with continuous cell lines from different tissues and transfected cells to investigate whether the pharmacological activity of metformin corresponds to the activity of organic cation transporters. We extended these studies to studies in primary hepatocytes and *in vivo* in *Oct1+/+* and *Oct1-/-* mice. Finally, we performed cellular and clinical studies determining the effect of genetic polymorphisms of OCT1 in the response to metformin. We observed the following: (i) In the cell lines, by controlling intracellular concentrations of metformin, Oct1 was an important determinant of metformin action. (ii) In primary cultures of hepatocytes from mice, eliminating functional Oct1 reduced the response to metformin, and in vivo in mice, Oct1 was required for metformin to therapeutically lower blood glucose levels. (iii) In humans, *OCT1* polymorphisms modulated cellular and clinical response to metformin. Collectively, our studies suggest that Oct1 mediates the first step in the response pathway

of metformin and that genetic variation in OCT1 may modulate response to metformin in humans (Figure 3.17).

Our results have important implications for the tissue specific effects of metformin. Although AMPK is ubiquitously expressed (27), the *in vivo* effects of metformin primarily have been ascribed to decreased hepatic gluconeogenesis and increased glucose uptake in skeletal muscle, both of which involve AMPK activation (4, 7, 28). We observed that the activation of AMPK by metformin was enhanced in cell lines (or transfected cells) that exhibited OCT activity, suggesting that the tissue specific action of metformin may be related to expression of influx transporters such as OCTs that can deliver metformin intracellularly. Our results differ from those of Huypens *et al.* who observed that metformin enhanced AMPK phosphorylation in 3T3-L1 cells (29). This discrepancy may be related to different experimental conditions. Huypens *et al.* used high metformin concentrations and prolonged exposure times which may have enhanced entry of metformin into the cells through passive diffusion resulting in sufficiently high intracellular drug concentrations for AMPK activation (29).

Our studies in *Oct1+/+* and *Oct1 -/-* mice (Figures 3.8-3.12) clearly demonstrated that Oct1 is a major determinant of the hepatic effects of metformin. This transporter-mediated tissue specific targeting of metformin may restrict the potential for unwanted effects in other tissues. Targeting influx transporters in the liver and skeletal muscle may be important in the design of other anti-diabetic agents to treat Type 2 diabetes. Further, our studies demonstrate that Oct1 plays a role in the primary therapeutic effect of metformin in lowering fasting blood glucose levels, suggesting that these effects may be mediated centrally in the liver. Previously, Wang *et al.* observed that Oct1 mediated the

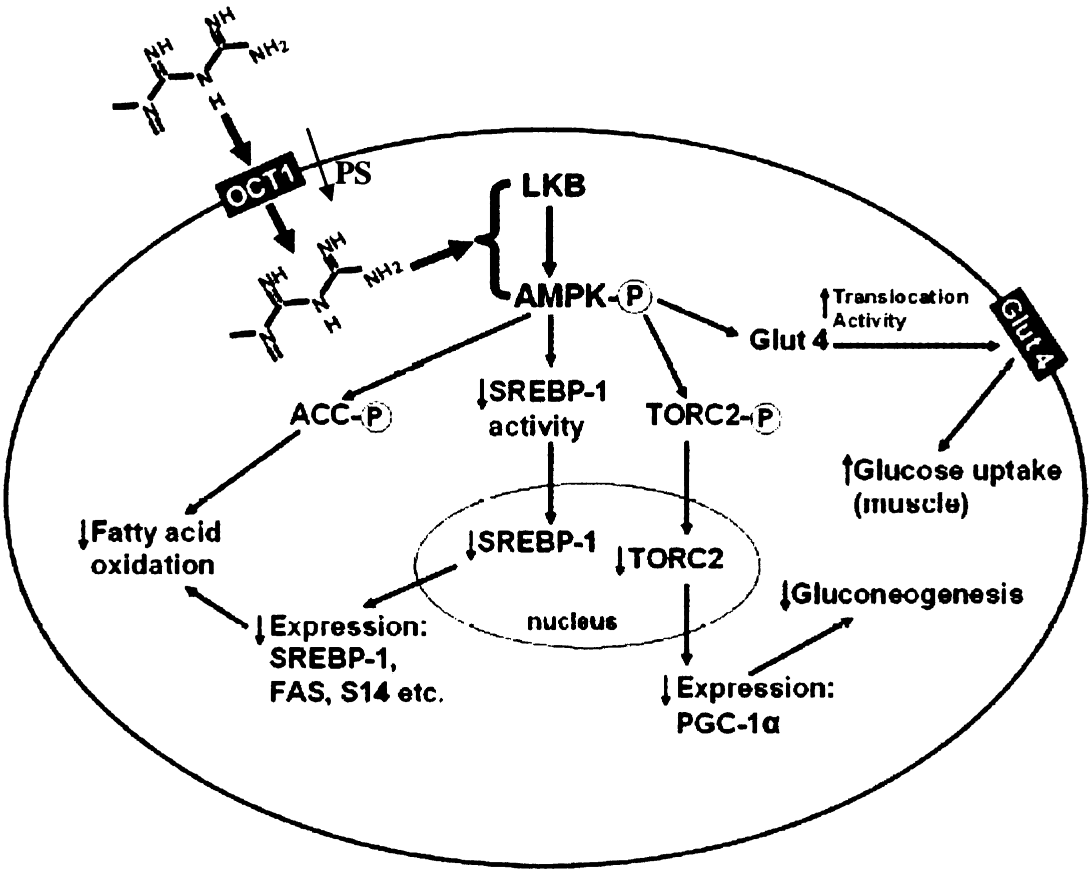

Figure 3.17. Mechanism of metformin action in cells. By controlling the intracellular concentrations, Oct1 is a direct determinant of metformin pharmacological effects in the liver. Passive diffusion (PS) contributes to the hepatic uptake of metformin to a much less extent. Other transporters may control metformin uptake into other tissues such as skeletal muscle. Factors such as genetic variation in transporter genes may alter transporter activity and thus metformin response.

major adverse effect of metformin, lactic acidosis, in the liver (30). We and Wang *et al.* (11) did not observe a significant difference in levels of metformin in skeletal muscle between *Oct1-/-* and *Oct1+/+* mice, suggesting that the uptake of metformin in skeletal muscle may involve another transporter.

In our *in vitro* studies, phosphorylation of AMPK and ACC by metformin was not abolished but substantially reduced in *Oct1-/-* hepatocytes in comparison to that in *Oct1+/+* hepatocytes (Figure 3.6B). Similarly, the oral treatment of metformin still resulted in a discernable increase in phosphorylation of AMPK and ACC in the livers of *Oct1-/-* mice (Figure 3.11). These data suggest that certain metformin-stimulated phosphorylation of AMPK and ACC is independent of Oct1 function. Passive diffusion and other transporters such as Oct2, which has a low level of expression in the liver (31), may account for the none-Oct1-mediated phosphorylation by metformin. Alternatively, metformin may initiate its pharmacological effects at an extracellular target site, such as a receptor, without crossing the membrane. It should be noted that the non-Oct1-mediated phosphorylation may not contribute significantly to metformin therapy because the glucose production inhibition and the glucose-lowering effect by metformin were abolished in *Oct1-/-* hepatocytes (Figure 3.8) and in *Oct1-/-* mice (Figure 3.12) respectively.

Clinically, there is enormous variation in response to metformin and the drug is generally combined with other agents such as sulfonylureas to treat diabetes. Data from a few clinical trials indicate that greater than 36% of patients receiving metformin monotherapy do not achieve acceptable control of fasting glucose levels (16, 17). Our data suggest that genetic variation in OCT1 may contribute to variation in response to

metformin. In cellular studies, we observed that seven of the twelve polymorphisms of OCT1 exhibited reduced transport of metformin (Figure 3.13). Previous studies from this laboratory using the model organic cation, MPP^+, demonstrated that four of these seven variants also had reduced activity (13). S14F was previously shown to exhibit an increased uptake of MPP^+, whereas it displayed a reduced uptake of metformin in this study related to a reduction in its V_{max} for metformin (Figure 3.13B, Table 3.3). Two other OCT1 variants, S189L and 420del, exhibited normal uptake of MPP^+ previously, whereas in this study both variants also exhibited a reduced uptake of metformin, related to a decrease in their V_{max} values (Figure 3.13B, Table 3.3). These data underscore the findings in the meta-analysis of Urban *et al.* who showed that transporter polymorphisms may interact differently with different substrates (32). It is noteworthy that 420del is a common polymorphism of OCT1, with an allele frequency of about 20% in individuals of European descent (13). Qualitatively, phosphorylation of ACC seemed to correspond with the level of uptake of metformin in the cell lines expressing the variants. That is, cell lines expressing reduced function variants had reduced phosphorylation of ACC. For AMPK, we observed that in cell lines expressing the loss of function OCT1 variants, AMPK-phosphorylation was reduced; however, in cell lines expressing decreased function variants, the effects on AMPK phosphorylation were variable. These discrepancies may be explained by the fact that our experiments were not sufficiently quantitative to detect subtle differences in AMPK phosphorylation by metformin. Alternatively, it is possible that cell lines over-expressing decreased function variants of OCT1 may take up sufficient quantities of metformin to produce maximal effects on the phosphorylation of AMPK.

Our clinical studies were focused on four of the OCT1 variants, R61C, G410S, 420del, and G465R, all of which exhibited reduced function in the cellular assays. Although no effect of OCT1 genotype on baseline OGTT was observed, significant effects of OCT1 genotype on OGTT were observed after metformin treatment (Figure 3.16). However, our study was limited as we used healthy volunteers who were not diabetic. Further studies in diabetic patients are needed to determine whether genetic variants of OCT1 may provide a basis for personalizing selection or dosing of metformin to achieve a maximal benefit for patients.

It is noteworthy that variation in the renal clearance of metformin also has a strong genetic component (33). In a previous study, genetic variation in OCT2, an OCT1 paralog expressed in abundance in the kidney, was found to alter metformin uptake kinetics (34). Further clinical studies are thus warranted to examine metformin disposition and response among individuals with different OCT2 genotypes. In addition, our results for metformin may be extended to other drugs that rely on transporters for disposition and/or targeting. For example, oxaliplatin, an important anti-cancer drug, has recently been well characterized as a substrate of both OCT1 and OCT2 (35). Patients with different OCT1 or OCT2 genotypes may respond differently to oxaliplatin chemotherapy.

In conclusion, the present study demonstrated that an uptake transporter, Oct1, is required for the anti-diabetic efficacy of metformin. The study provides proof of concept that genetic variation in OCT1 may associate with response to metformin.

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CHAPTER 4

EFFECTS OF OCT1 FUNCTION ON THE PHARMACOKINETICS AND PHARMACODYNAMICS OF METFORMIN

Introduction

Response to a drug is determined by both its pharmacokinetic and pharmacodynamic properties. Until recently, pharmacokinetic studies have focused largely on drug metabolizing enzymes. However, it is becoming increasingly clear that membrane transporters are also important determinants of pharmacokinetics (1). For example, the efflux transporter, P-glycoprotein, plays a role in drug absorption and distribution (2). Transporters that serve primarily in intracellular influx, such as the organic anion transporting polypeptide 2 (OATP1B1), also appear to be determinants of the pharmacokinetic properties of many drugs including fexofenadine (3), pitavastatin (4) and nateglinide (5). Organic cation transporter 1 (OCT1), an influx transporter encoded by *SLC22A1*, interacts with a variety of structurally diverse compounds including clinically used drugs such as the anti-cancer drug oxaliplatin (6), the anti-diabetic drug metformin (7, 8) and the anti-hypertensive drug pindolol (9). However, the role of OCT1 in the pharmacokinetics of drugs in humans remains to be determined.

The effect of drug transporters on pharmacokinetics may be easily translated to an effect on pharmacodynamics. In particular, by controlling systemic drug concentrations and drug concentrations in tissues and in the vicinity of receptors and other drug targets, membrane transporters are also determinants of pharmacodynamics. It is thus intriguing

to evaluate the role of drug transporters such as OCT1 in both pharmacokinetics and pharmacodynamics.

Metformin, a widely used anti-diabetic agent (10), is a substrate of OCT1 (7, 8). The drug is eliminated from the body exclusively by the kidney without any significant metabolism *in vivo* (11), excluding any confounding effects on pharmacokinetics by drug-metabolizing enzymes. Following intravenous doses, *Oct1-/-* mice and *Oct1+/+* mice have similar systemic pharmacokinetic properties (7). However, the role of OCT1 in metformin pharmacokinetics remains to be determined following an oral dose of the drug, given the fact that gastrointestinal absorption of metformin is incomplete with an oral bioavailability of 40 to 60%, and slow absorption is the rate-limiting factor in metformin disposition (11).

Human OCT1 has a wide tissue distribution with primary expression in the liver and lower levels in other tissues, including intestine and skeletal muscle (12). Metformin appears to ameliorate hyperglycemia by reducing hepatic glucose production and gastrointestinal glucose absorption and by improving glucose utilization in skeletal muscle (10). The coincident tissue expression patterns of OCT1 with the sites of action of metformin suggest that OCT1 may be a determinant of the pharmacodynamics metformin.

We and others have demonstrated that human *OCT1* exhibits large variation in the coding region (13-15). A number of non-synonymous polymorphisms have been found to have reduced transport for model OCT1 substrates and/or metformin. Some of the reduced function variants are common in populations. For example, in European Americans, OCT1-420del has an allele frequency of 18.5% and OCT1-R61C has a

frequency of 7.2% (15). In addition, several reduced function or non-functional variants have been identified with ethnic-specific allele frequencies of greater than 1% (e.g., OCT1-P341L, OCT1-G401S and OCT1-G465R). The polymorphisms of OCT1 provide a tool to study the *in vivo* role of OCT1 in pharmacokinetics and pharmacodynamics in humans. Given that there is considerable variation in response to metformin among diabetic patients (16, 17), it is reasonable to propose that polymorphisms of OCT1 may contribute to this variation.

In this study we first determined the pharmacokinetics of metformin after oral doses in *Oct1-/-* mice and *Oct1+/+* mice. Further, we investigated the effects of *OCT1* genotype on the pharmacokinetics of metformin in healthy human volunteers. Glucose tolerance test (GTT) and other biomarker measurements were performed to evaluate metformin pharmacodynamics in mice and in humans. Part of the data has been presented in Chapter 3.

Materials and Methods

Mice. *Oct1-/-* mice were generated as described elsewhere (see Chapter 3). The animals used in all experiments were age-matched *Oct1-/-* and *Oct1+/+* (wild-type) mice, of comparable mixed genetic background (on average 97% FVB, 1.5% 129/OLA and 1.5% C57BL6), between 10 and 14 weeks of age. All animals were housed in a virus-free, temperature-controlled facility on a 12-hour light-dark cycle. They received either standard mouse food or a high-fat diet (diet D12492, Research Diets, New Brunswick, NJ) and water *ad libitum*. All experiments on mice were approved by the

Institutional Animal Care and Use Committee of University of California at San Francisco (UCSF).

Metformin pharmacokinetics in mice. Ten week old male mice were fasted for 12 hours, then given an oral gavage dose of 15 mg/kg metformin in saline with 0.2 μCi/g of ^{14}C-metformin, and placed in metabolic cages for 24 hours. The food was re-introduced four hours after metformin treatment. Blood samples were collected before metformin treatment (0 hr) and at 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10, 12, and 24 hours after metformin treatment by tail bleeding into heparinized micro-hematocrit capillary tubes (Fisher, Pittsburgh, PA). Urine and feces were collected from tubes attached to the cages. Metformin in blood, fecal homogenates, and urine was measured by scintillation counting. The pharmacokinetic parameters were obtained by noncompartmental analysis using WinNonlin 4.0 (Pharsight Corporation, Mountain View, CA).

Effect of metformin on glucose tolerance test (GTT) in mice. To study the effect of Oct1 function on metformin pharmacodynamics, we performed GTTs on age-matched male *Oct1-/-* and *Oct1+/+* mice treated with metformin. Eight week old mice were fed a high fat diet for four weeks. The mice were then fasted for 16 hours before a GTT. For the GTT, glucose (2 g/kg) was administered intraperitoneally, and blood was collected before (0 min) and at 15, 30, 60, 90, and 120 min after glucose administration. Plasma was isolated as described above, and glucose concentration was measured using the glucose oxidase assay kit (Sigma). The mice continued to be fed the high-fat diet for

two more weeks. The 14 week old mice were then injected intraperitoneally with 50 mg/kg metformin, a dosage approximating maximal doses used to treat diabetic patients, in the morning for five days. At 6:00 PM on day four, the mice were fasted for 16 hours. GTT was conducted as described above on day five.

Healthy human volunteers. Healthy human volunteers in the present study were recruited from those who participated in another study, SOPHIE (Study of Pharmacogenetics in Ethnically Diverse Populations). Subjects in the SOPHIE cohort range between the ages of 18 and 40 and have been evaluated to be healthy on the basis of medical history provided by a study questionnaire. All SOPHIE participants have signed prior consent for genetic testing and have given permission to be contacted about their willingness to participate in related research studies. To be eligible for the present study, subjects could not be taking any medications other than vitamins. Individuals with anemia (hemoglobin < 12 g/dL), elevated liver enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyltransferase) to greater than double the respective normal value, or elevated creatinine concentrations (males $\geq$ 1.5 mg/dL, females $\geq$ 1.4 mg/dL), were excluded. Women of childbearing age were asked to provide a urine sample to confirm a negative pregnancy test before the study.

No data are currently available regarding the effect of ethnicity on metformin disposition and response. In the previous study using the OCT1 model substrate, MPP^+, we found that the common variants of OCT1 with reduced or no function have ethnic-specific allele frequencies (1% - 8%) (15). Interestingly, those variants occur primarily in Caucasians. Therefore, we first genotyped Caucasian DNA samples in SOPHIE for three

common reduced or loss of function variants: OCT1-R61C, OCT1-G401S or OCT1-G465R (see genotyping below). Our subsequent recruitment from SOPHIE was limited to healthy male or female Caucasian volunteers who had any of the three reduced or loss of function variants. During the study, the participants were also genotyped for OCT1-420del (see results below). For clarity in this study, individuals who carried any of the four polymorphisms, OCT1-R61C, OCT1-G401S, OCT1-420del and OCT1-G465R, are referred to as individuals with an OCT1-variant, and those who had the reference allele at all four positions are termed individuals with OCT1-reference alleles.

Genotyping. OCT1-R61C and OCT1-G465R were genotyped by a TaqMan assay. The reaction mixture consisted of 1 µL of 2X TaqMan Master Mix, 0.05 µL of Assay Mix (yielding a final primer concentration of 900 nM and final probe concentration of 200 nM), 0.95 µL of double-distilled H_2O, and 2 ng of DNA pre-dried in the plate. The cycling conditions were: 92^0C 10 minutes, 50 cycles of 92^0C for 15 seconds and 60^0C for 1 minute, and a 4^0C terminal hold. The reaction was run on ABI 7900HT.

OCT1-G401S and OCT1-420Del were genotyped by sequencing the exons. The PCR mixture consisted of 0.2 µL of 10X PlatTaq PCR Buffer, 0.2 µL of 2 µM dNTP mix, 0.06 µL of 50 mM $MgCL_2$, 0.02 µL of 5U/µL Platinum Taq (Invitrogen), 0.4 µL of 5 M Betaine, 0.04 µL of 100% DMSO, 1.08 µL of double-distilled H_2O, and 10 ng of genomic DNA pre-dried in the plate. The PCR cycling conditions were: 95^0C for 5 minutes; 10 touchdown cycles of 94^0C for 20 seconds, 61^0C (-0.5^0C each cycle) for 20 seconds, and 72^0C for 45 seconds; 35 cycles of 94^0C for 20 seconds, 56^0C for 20 seconds,

and 72^0C for 45 seconds; 72^0C for 10 minutes; and a 4^0C terminal hold. The PCR products were cleaned up by adding 0.25 μL of 1 U/μL SAP (shrimp alkaline phosphatase), 0.025 μL of 10 units/μL Exonuclease I, and 1.725 μL of double-distilled H_2O to the 2 μL PCR reaction for a 4 μL final volume. The cleanup conditions were: 37^0C for 60 minutes, 95^0C for 15 minutes, and a 4^0C terminal hold. The sequencing mixture consisted of 0.5 μL of BigDyev3.1, 0.75 μL of 5X sequencing dilution buffer, 2 μL of 1 μM primer, 0.75 μL of double-distilled H_2O, and 1 μL of PCR product. The mixture was cycled as: 94^0C for 1 minute; 30 cycles of 94^0C for 10 seconds, 55^0C for 5 seconds, and 60^0C for 4 minutes; and 4^0C for the terminal hold. The sequencing products were cleaned up using Millipore Montage, run on ABI 3730XL and analyzed with Sequencher Version 4.5. The primers for genotyping are summarized in Table 4.1.

Clinical study procedures. The study protocol was reviewed and approved by the Committee on Human Research at UCSF. The general study design has been described elsewhere (see Chapter 3). In brief, after informed consent was obtained, healthy individuals with known OCT1 genotypes were recruited into this open label study. To reduce variation in the results of the oral glucose tolerance test (OGTT), our primary determinant of metformin pharmacodynamics, participants were advised to maintain stable activity levels (without periods of strenuous exercise) for seven days and carbohydrate intake at 200-250 g/day for three days before the formal study. Subjects were admitted (Day 0) to the General Clinical Research Center (GCRC) at San Francisco General Hospital (SFGH), and remained at this center for the duration of the study. In the morning of Day 1, a 3-hour oral glucose tolerance test (OGTT, 75 g glucose) was

Table 4.1. Genotyping primers for OCT1-R61C, OCT1-G465R, OCT1-G401S and OCT1-420Del.

Variant	Primers (5'→ 3')
R61C	Forward-GCCTTTGCGCCCATCTG
	Reverse-CTCCGCAGGGCTCCAG
	VIC Probe-CACAGCGCTGGCTC
	FAM Probe-CACAGCACTGGCTC
G465R	Forward-GTGAATCACAGAATTATCGTATTTTTTGTCCT
	Reverse-GTCACACAGGGAGGAACACA
	VIC Probe-TCAGGAACCTCGGAGTGA
	FAM Probe-CAGGAACCTCAGAGTGA
G401S	Forward-TTTCTTCAGTCTCTGACTCATGC
	Reverse-TCCCCACACTTCGATTGC
420Del	Forward-TTTCTTCAGTCTCTGACTCATGC
	Reserve- TCCCCACACTTCGATTGC

conducted, and blood samples were collected at 0, 15, 30, 45, 60, 90, 120, and 180 min after glucose administration. Subjects were then dosed with 1000 mg metformin in the evening followed by a dose of 850 mg on the morning of the second day, two hours before the initiation of a second OGTT.

For metformin pharmacokinetics, additional blood samples were collected at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10, 12, and 24 hours after the second metformin dose for determination of plasma metformin concentrations. Standardized meals were provided starting five hours after metformin administration (at noon). Following the glucose dose, volunteers were asked to drink eight ounces of water every four hours to maintain urine flow and pH. Urine samples were collected between the following time points: 0-2, 2-4, 4-8, 8-12, and 12-24 hours after the second metformin dose. The volume and pH of urine were recorded for each interval and 20 mL of the urine was then stored at –20°C for analysis of metformin content.

Analytical methods for clinical samples. Metformin concentrations in plasma and urine were assayed by highly specific and sensitive liquid chromatography–tandem mass spectrometry methods (LC/MS/MS). To prepare the samples for LC/MS/MS analysis, an aliquot of the clinical plasma or urine samples was mixed with acetonitrile in the presence or absence of the internal standard, propranolol. The mixture was vortexed for one minute and then centrifuged for ten minutes at 3000 rpm. An aliquot of the supernatant was transferred to an autosampler vial and 3 μl was injected onto the column in a 4^0C autosampler. The mobile phase consisted of 80% acetonitrile, 20% double-distilled water, 0.5% 2 M ammonium acetate aqueous solution and 0.05% acetic acid

(vol/vol). The quantification limit was 10 ng/mL for plasma and 100 ng/ml for urine. Both the intra-day and inter-day coefficients of analysis variation were less than 10%. Plasma levels of insulin, triglyceride, and total cholesterol were assayed by the Clinical Laboratory in San Francisco General Hospital (SFGH). Plasma glucose and lactate were measured by the Core Facility of the GCRC in SFGH.

Clinical pharmacokinetics. The concentration-time profile of metformin was evaluated by noncompartmental analysis (WinNonlin 4.0, Pharsight Corporation). The subjects received two doses of metformin to maximize the pharmacodynamic effects. The second dose (1000 mg) was received 12 hours after the first dose (850 mg). Metformin pharmacokinetics was reported to be linear with increasing doses up to 2500 mg. In this study, we calculated the pharmacokinetics of metformin from the plasma and urine concentrations after the second dose, with consideration of the contribution of residual metformin from the first dose. The peak plasma concentration (C_{max}) and time to C_{max} (T_{max}) were directly determined from the plasma concentration-time profile. The elimination rate constant (k_e) was estimated from the slope of the best-fit line determined by linear regression analysis of the log-linear part of the concentration-time curve. The elimination half-life ($T_{1/2}$) was calculated by the equation $T_{1/2} = \ln 2/k_e$. The apparent area under the plasma concentration-time curve (AUC_A) after the second dose was calculated by the linear trapezoidal rule for the rising phase of the plasma concentration–time curve, by the log-linear trapezoidal rule for the descending phase, and extrapolation to infinity calculated as division of the last measured concentration by k_e. The contribution of residual metformin from the first dose was estimated from the plasma concentration at

time 0. The residual AUC (AUC_R) was calculated by division of the concentration at time 0 by k_e. Thus the AUC for the second dose (AUC_B) was calculated by subtraction of AUC_R from AUC_A. Renal clearance (CL_R) was calculated for metformin by dividing Ae (metformin excreted in the urine from 0-24 hours) by $AUC_{0\text{-}24hr}$. Metformin apparent oral clearance (CL_{oral}) was calculated by dividing the second dose by AUC_B. The apparent oral volume of distribution (V_{oral}) was calculated by the equation $V_{oral} = CL_{oral} / k_e$.

Clinical pharmacodynamics. The glucose-lowering effects of metformin in OGTT were significantly lower in individuals carrying reduced function polymorphisms of OCT1 (see Chapter 3). We also measured the plasma levels of insulin, lactate, triglycerides, and cholesterol for the blood samples collected during OGTTs. In the present study, we compared these measurements before and after metformin treatment between individuals who carry the decreased or non-functional OCT1 variants and those who carry OCT1-reference alleles.

Statistical analysis. Unless indicated, the data are presented as mean ± standard deviation (SD). The pharmacokinetic and pharmacodynamic variables between the reference and mutant genotypes were compared using one or two tailed *Student's* t-test. The level of statistical significance was set at $P < 0.05$.

Results

Metformin pharmacokinetics in *Oct1-/-* and *Oct1+/+* mice. The *Oct1-/-* (n = 6) and *Oct1+/+* mice (n = 5) were dosed with 15 mg/kg metformin through oral gavage. The 24-hour plasma concentration-time profile was similar in *Oct1-/-* and *Oct1+/+* mice. However, the blood metformin concentrations tended to be higher in *Oct1-/-* mice than in *Oct1+/+* mice. This was most obvious at the absorption phase (0 - 1.0 hour) of metformin (Figure 4.1). Consistent with the tendency of blood concentration difference, *Oct1-/-* mice had a significantly greater metformin AUC during the absorption phase (P = 0.05, Table 4.2). Moreover, *Oct1-/-* mice tended to have a greater metformin AUC from 0 to 24 hours and a smaller oral volume of distribution (P = 0.07 and 0.09 for AUC and V/F respectively, Table 4.2). In addition, *Oct1-/-* mice seemed to excrete slightly less metformin in the urine compared to *Oct1+/+* mice. However, the difference did not reach statistical significance.

Effect of metformin on glucose tolerance test (GTT) in *Oct1-/-* and *Oct1+/+* mice. In the previous study, we demonstrated that the effect of metformin on fasting glucose concentration was abolished in *Oct1-/-* mice fed a high fat diet (see Chapter 3). In this study, we performed GTT on age-matched *Oct1-/-* and *Oct1+/+* mice before and after metformin treatment to further evaluate the effect of Oct1 function on metformin pharmacodynamics. The *Oct1-/-* and *Oct1+/+* mice were fed a high-fat diet for four to six weeks to induce a diabetic status. We performed the first GTT on the mice receiving the high-fat diet for four weeks without metformin treatment. We observed similar plasma glucose levels and areas under the glucose concentration-time curves (AUC) after

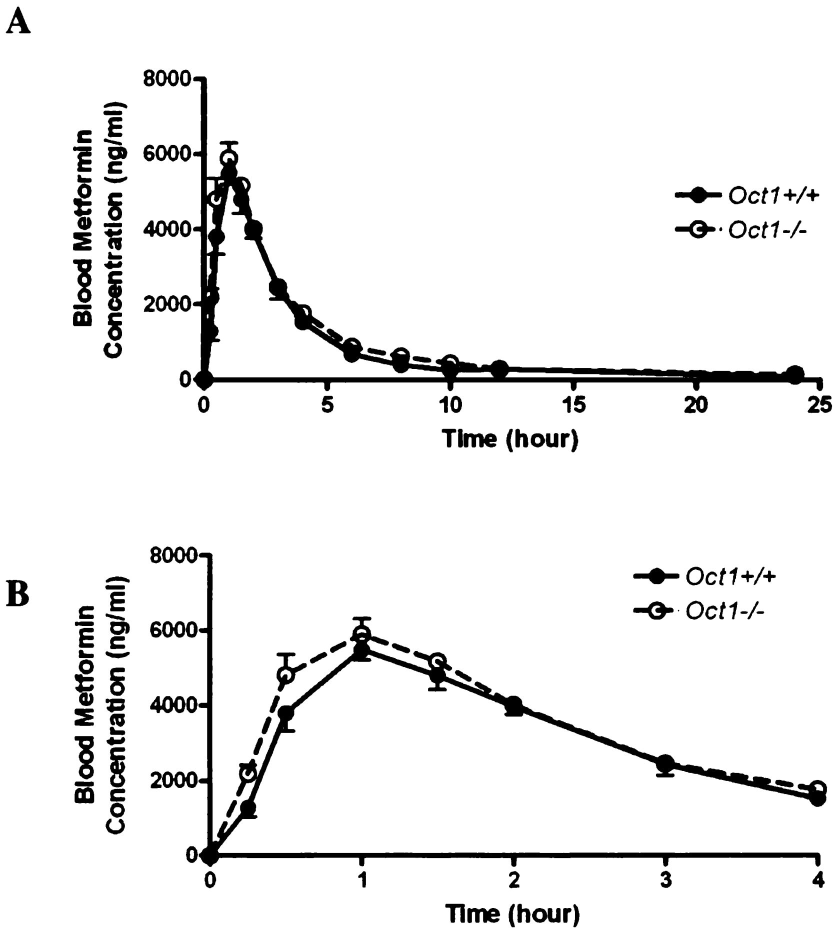

Figure 4.1. The blood concentration-time curves of metformin after an oral dose in *Oct1+/+* mice (n = 5) and *Oct1-/-* (n = 6) mice. The mice were given an oral dose of metformin (15 mg/kg containing 0.2 mci/kg of ^{14}C-metformin), approximating the single dose of 1000 mg in humans. Data represent mean ± SE. **(A)** The blood concentration-time curve from 0 to 24 hours. The data of eight mice (four of each Oct1 genotype) have been presented in Chapter 3, Figure 3.7A. **(B)** The blood concentration-time curve for the first four hours after metformin administration. The difference in AUC of the absorption phase (0 – 1 hour) is barely significant between *Oct1+/+* and *Oct1-/-* mice (3.1 ± 0.25 *vs.* 4.0 ± 0.33 μg∗hour/mL, $P = 0.05$).

Table 4.2. Metformin pharmacokinetic parameters from *Oct1*+/+ and *Oct1*-/- mice. The mice were given an oral dose of metformin (15 mg/kg containing 0.2 mci/kg of ^{14}C-metformin), approximating the single dose of 1000 mg in humans. The radioactivity in blood was determined and converted to mass amounts.

	Oct1+/+ (n=5)		*Oct1*-/- (n=6)	
	Mean	SD	Mean	SD
$T_{1/2}$ (h)	5.5	2.0	5.4	1.7
$AUC_{0\text{-}1hr}$ (μg*h/mL)	3.1	0.57	4.0*	0.73
$AUC_{0\text{-}24hr}$ (μg*h/mL)	19.0	1.9	21.8	2.5
V/F (mL)	356	200	187	75
CL/F (mL/h)	29.7	6.0	23.6	4.5
Urine recovery (%)	60	7	50	13
Feces recovery (%)	30	10	29	3

Note: $T_{1/2}$: half life; AUC: area under the curve of blood concentration-time of metformin; V/F: oral volume of distribution (volume of distribution over oral bioavailability); CL/F: oral clearance (clearance over oral bioavailability).

*$P = 0.05$ compared with *Oct1*+/+ mice.

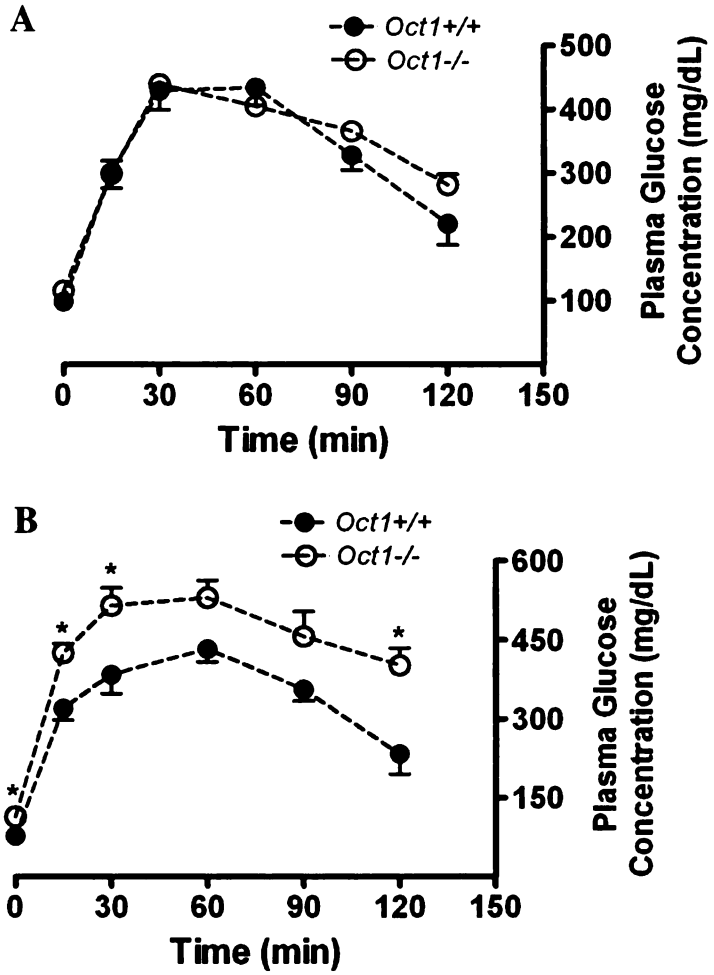

Figure 4.2. *Oct1* deficiency is associated with different responses to metformin in mice. **(A)** The time course of plasma glucose concentrations for a baseline glucose tolerance test (GTT) without metformin treatment in *Oct1*+/+ (n = 5) and *Oct1*-/- mice (n = 5). The eight week old mice were fed a high-fat diet for four weeks before the GTT. **(B)** The time course of plasma glucose concentrations for GTT after metformin treatment in the same mice studied in A. The mice continued to be fed the high-fat diet for two weeks before this second GTT. The data are expressed as mean ± SE; **P* < 0.05 compared with *Oct1*+/+ mice, unpaired *Student's* t-test.

GTT in *Oct1-/-* and *Oct1+/+* mice (AUC: 41100 ± 5670 *versus* 43100 ± 2560 min·mg/dL, Mean ± SD, *P* = 0.490; Figure 4.2A). The mice continued to be fed the high-fat diet for two more weeks. However, with five days of metformin treatment, *Oct1-/-* mice had significantly higher plasma glucose levels for most of the sampling time points (0, 15, 30, and 120 min) during the 120-min GTT than did *Oct1+/+* mice (Figure 4.2B). Thus AUC of glucose was significantly greater for *Oct1-/-* mice as compared to *Oct1+/+* mice (41200 ± 5270 *versus* 51700 ± 6900 min·mg/dL, *P* = 0.026).

OCT1 genotyping. We obtained 208 Caucasian DNA samples from the SOPHIE cohort. Of the 208 samples, 30 (14.4%) were heterozygous for OCT1-R61C, 9 (4.3%) heterozygous for OCT1-G401S, and 10 (4.8%) heterozygous for OCT1-G465R. One subject (0.005%) was homozygous for OCT1-R61C. Interestingly, no subject was found to have more than one of the three polymorphisms. The observed frequencies were in Hardy-Weinberg equilibrium, and comparable to those observed previously (13, 15).

The clinical study was initially based on the previous cellular characterization (15) and designed to assess the effects of OCT1-R61C, OCT1-G401S and OCT1-G465R on metformin pharmacokinetics and pharmacodynamics. We thus recruited from the SOPHIE project ten volunteers who carried the variant allele and ten volunteers who did not have any of the three variants. In further cellular studies, we identified another common OCT1 variant (420Del) that exhibited a reduced metformin uptake (see Chapter 3). All of the participants in this clinical study were thus genotyped for OCT1-420del as well. Of the 20 volunteers, 5 (25%) volunteers were found to be OCT1-420del

heterozygotes. Three of the five were also heterozygous for G465R. The characteristics and genotypes for the subjects are summarized in Chapter 3.

Effects of OCT1 genotype on metformin clinical pharmacokinetics. The OCT1 genotype had a small but significant effect on the pharmacokinetics of metformin (Figures 4.3, Table 4.3). The 24-hour plasma concentration-time profile was similar between the individuals who carried the variants and those who did not. Consistent with the results from the mice, plasma metformin concentrations tended to be higher in the individuals with an OCT1-variant than those only with OCT1-reference. Metformin concentrations at 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 6.0, 8.0, and 10 hr were significantly higher in the individuals carrying the variants than in those carrying OCT1-reference ($P < 0.05$). There was no difference in the time (T_{max}) to the maximal plasma concentration (C_{max}). However, individuals who carried the variants had a significantly higher C_{max} than those who did not carry the variants ($P = 0.004$). Metformin AUC was significantly greater in individuals with an OCT1-variant than those only with OCT1-reference ($P = 0.01$). The oral volume of distribution was significantly lower in individuals with the variants compared to those with OCT1-reference (35% lower, $P = 0.003$). Whereas individuals with OCT1-reference had a significantly higher oral clearance (18.2% higher, $P = 0.013$), the renal clearance was similar between the two OCT1 genotype groups ($P = 0.746$). No other pharmacokinetic parameters were found to be significantly different between the two genotype groups.

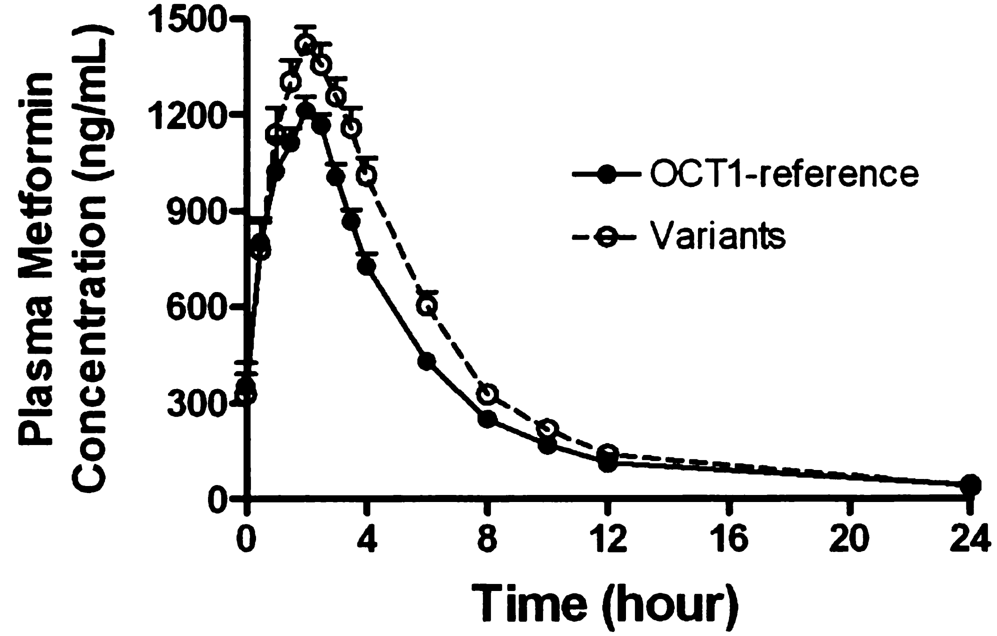

Figure 4.3. The plasma concentration-time curves of metformin after oral administration of metformin in healthy individuals who carry OCT1 variants (n = 12) and those who carry only OCT1-reference alleles (n = 8). The individuals were given two doses of metformin. The first dose of 850 mg was given at 6 pm on study day 1 and the second dose of 1000 mg at 6 am on study day 2. Blood samples for the pharmacokinetic analysis were then drawn up to 24 hours after the second dose. Shown here are the plasma metformin concentration-time curves after the second oral dose of metformin. Data represent mean ± SE.

Table 4.3. Metformin pharmacokinetic parameters from healthy individuals who carry OCT1-reference alleles (OCT1-reference) and those who carry an OCT1 variant (variants). The individuals were given two doses of metformin. The first dose of 850 mg was given at 6 pm on study day 1 and the second dose of 1000 mg at 6 am on study day 2. The first blood sample (0 hour) was drawn immediately before the second dose. Blood samples for the pharmacokinetic analysis were then drawn up to 24 hours.

	OCT1-reference (n=8)		Variants (n=12)	
	Mean	SD	Mean	SD
$T_{1/2}$ (hour)	7.3	2.3	5.8	1.2
T_{max} (hour)	1.9	0.52	2.2	0.72
C_{max} (μg/ml)	1.3	0.10	1.5*	0.19
AUC (hour*μg/L)	7700	970	9200*	1200
V/F (L)	1400	410	910*	200
CL/F (L/hour)	130	21	110*	15
CL_R (L/hour)	39	17	36	21

Note: $T_{1/2}$: half life; T_{max}: time to the maximal plasma concentration; C_{max}: maximal plasma concentration; AUC: area under the curve of plasma concentration-time of metformin; V/F: oral volume of distribution (volume of distribution divided by oral bioavailability); CL/F: oral clearance (clearance over oral bioavailability); CL_R: renal clearance.

*$P < 0.05$ compared with the OCT1-reference.

Effects of OCT1 genotype on metformin clinical pharmacodynamics. As described elsewhere, OCT1 genotype had a significant effect on OGTT in the healthy volunteers (see Chapter 3). We also measured the plasma levels of insulin, lactate, triglycerides and total cholesterol before and after metformin treatment (Figure 4.4). After glucose administration, plasma insulin levels immediately went up, peaked between 30 and 60 min, and then declined. Metformin tended to attenuate the rise in insulin levels immediately after glucose administration in both OCT1 genotype groups. Possibly because of large inter-individual variability, the insulin levels in the period after metformin administration were not significantly lower than those in the same period without metformin treatment for both groups. After metformin administration, insulin secretion was significantly greater in individuals carrying the OCT1 variants compared to those carrying the reference alleles (Insulin AUC: variants *vs.* wild-type, 8200 ± 4800 min·μIU/ml *vs.* 4400 ± 1900 min·μIU/ml, $P = 0.025$; Figure 4.4).

Metformin treatment increased plasma lactate levels during the OGTT in all individuals irrespective of OCT1 genotype (Figure 4.5). The two doses of metformin had no effect on the plasma levels of cholesterols and triglycerides (data not shown). In addition, no significant differences were found in the plasma levels of lactate, triglycerides and total cholesterols between the individuals with different OCT1 genotypes with and without metformin treatment.

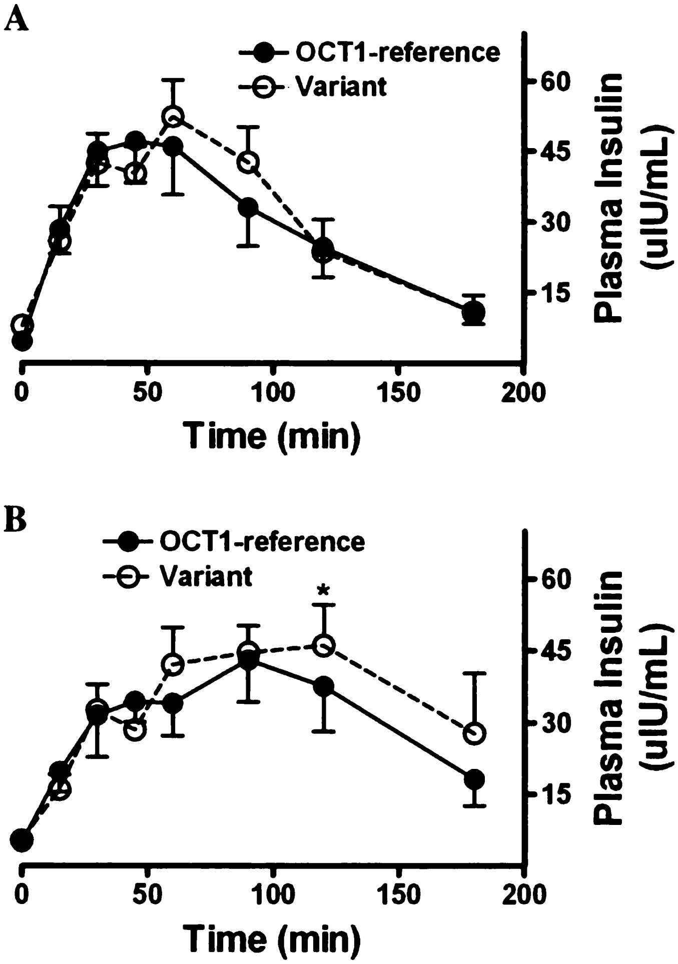

Figure 4.4. OCT1 genetic variants are associated with different responses to metformin in healthy individuals. **(A)** The time course of insulin levels during a base line oral glucose tolerance test (OGTT) without metformin treatment in healthy individuals carrying *OCT1*-reference alleles (n = 8) and those carrying at least one reduced-function variant allele of *OCT1* (n = 12). The data are expressed as mean ± SE. **(B)** The time course of insulin levels during the OGTT after metformin administration in the same healthy individuals studied in A. The data are expressed as mean ± SE; $*P < 0.05$ compared with individuals with only *OCT1*-reference alleles, unpaired *Student's* t-test.

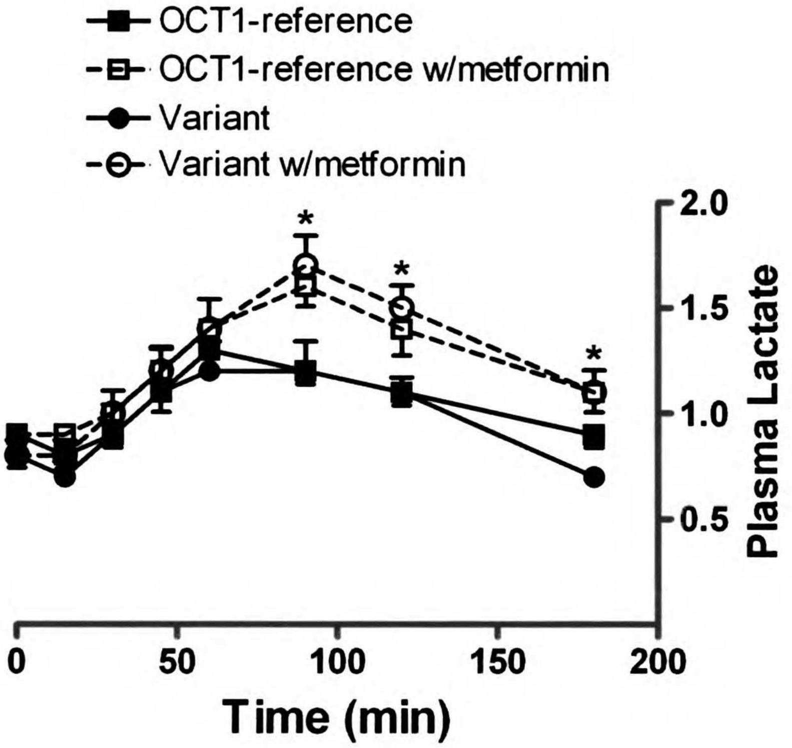

Figure 4.5. The lactate levels during glucose tolerance tests (OGTT) with and without metformin treatment in healthy individuals carrying *OCT1*-reference alleles (n = 8) and those carrying at least one reduced-function variant allele of *OCT1* (n = 12). The data are expressed as mean ± SE. *$P < 0.05$ compared with those without metformin treatment for each of the two genotype groups respectively, paired *Student's* t-test.

Discussion

This study provides evidence that OCT1 genotype and activity affects the pharmacokinetics of metformin. In particular, the pharmacokinetic properties of metformin including AUC, V/F and C_{max} were significantly different between the individuals who carried the reduced function variants, OCT1-R61C, G401S, 420del, or G465R and those who carried only OCT1-reference alleles. Similar trends were observed in *Oct1-/-* and *Oct1+/+* mice. Human OCT1 is mainly expressed in the liver (12, 18, 19). Hepatic uptake of metformin is dramatically reduced in *Oct1-/-* mice compared to *Oct1+/+* mice after metformin administration (7) (Chapter 3). The effects of OCT1 genotype on the pharmacokinetics of metformin, as evidenced by the smaller oral volume of distribution in *Oct1-/-* mice and in the individuals carrying the reduced function variants of OCT1, may be explained by differences in the distribution of the drug to the liver. Metformin is not metabolized in the body (11). We expect that, for OCT1 substrates that are extensively metabolized in the liver, OCT1 may be a critical determinant of their access to drug-metabolizing enzymes and therefore may have a larger effect on their pharmacokinetics and pharmacodynamics.

The renal clearance of metformin was similar between the individuals who carried the OCT1 variants and those who carried only reference alleles of OCT1. Interestingly, however, the oral clearance (CL/F) was significantly higher in the individuals who carried the reference alleles than in those who carried the variant alleles. Since individuals with OCT1 reference alleles may take up more metformin in the liver, the higher CL/F may be due to a higher clearance of metformin. It has been reported that the biliary excretion of the model organic cation, tetraethylammonium (TEA), was reduced

to about half in *Oct1-/-* mice compared to *Oct1+/+* mice (20). OCT1 is also expressed in on the basolateral membrane of the intestine (12) and therefore intestinal clearance of metformin may be reduced in individuals with reduced function variants of OCT1. Significantly reduced distribution of metformin in the intestine has been observed in *Oct1-/-* mice in comparison to *Oct1+/+* mice (7).

The effect of OCT1 activity on metformin pharmacokinetics was less pronounced in mice than in healthy individuals. We did not detect statistically significant differences in the pharmacokinetic properties of metformin between *Oct1-/-* and *Oct1+/+* mice, suggesting species differences in the role of OCT1 in metformin disposition. The greater effect of OCT1 on metformin pharmacokinetics in humans may be explained by higher expression levels of OCT1 in human liver in comparison to mouse liver. In humans, OCT1 is predominantly expressed in the liver, with low levels in other tissues (12, 18, 19). In rodents, Oct1 is expressed in equal abundance in the liver, kidney, and small intestine (21, 22). It is also possible that the role of Oct1 in metformin disposition in mice may be less because of the presence of functionally redundant transporters. Alternatively, metformin may have a lower affinity to mouse Oct1 in comparison to human OCT1. In addition, there may be differences between mice and humans in the activity of the transporters responsible for transporting metformin in other tissues such as intestine and kidney which are important for metformin disposition in the body.

In this study, we observed that *Oct1-/-* mice and individuals carrying the reduced function variants of OCT1 had a significantly lower pharmacodynamic response to metformin. In particular, we observed that the improvement of glucose tolerance by the drug was significantly reduced in *Oct1-/-* mice compared to *Oct1+/+* mice. The data are

consistent with those described in the previous chapter in healthy human volunteers with different OCT1 genotypes. In non-diabetic healthy subjects, it has been reported that metformin significantly attenuates the rise in immediate postprandial insulin levels (23). In this study, we observed that metformin tended to attenuate the rise in insulin levels immediately after glucose administration. In the absence of metformin, insulin secretion (reflected here by insulin AUC) in response to glucose administration was comparable between the two groups of different OCT1 genotypes. However, following metformin administration, insulin secretion was greater and lasted longer in individuals carrying the reduced function variants of OCT1. Our data indicate that OCT1 polymorphisms may be important determinants of the pharmacodynamics of metformin. As discussed in Chapter 3, further studies are required to explore the clinical significance of OCT1 polymorphisms in patients, particularly in diabetic patients receiving metformin therapy.

Interestingly, in the individuals carrying the OCT1 variants, the total insulin secretion was significantly greater during the OGTT with metformin treatment than without metformin treatment. The reason is unknown. One possibility is that metformin may have an effect on insulin secretion. Alternatively, the glucose tolerance in the individuals carrying the OCT1 variants is somewhat less in comparison to those carrying the reference alleles. We observed that before metformin treatment, the individuals carrying the OCT1 variants tended to have higher insulin levels in response to glucose administration. Besides its role as a drug transporter, OCT1 may have a physiological role with respect to glucose disposition and insulin sensitivity. Our recent preliminary data from mice are consistent with this hypothesis (see Chapter 5).

Lactic acidosis is the most severe, dangerous side effect in patients receiving metformin (10, 11). When mice were given metformin, blood lactate concentrations significantly increased in the wild-type mice, whereas only a slight increase was observed in *Oct1-/-* mice (24). In this study, metformin treatment resulted in elevated plasma lactate concentrations in all of the individuals studied irrespective of OCT1 genotype. Clinically, not all cases of metformin-related lactic acidosis are associated with high concentrations of metformin in the plasma (25), therefore it is possible that OCT1 genotype is not a determinant of lactate levels in the plasma. It is also possible that the doses used in this study were too low. Substantially greater doses per body weight were used in the studies in *Oct1-/-* mice (24).

Metformin can improve lipid metabolism in obese and diabetic patients, which is reflected by reduced plasma levels of triglycerides, cholesterol and free fatty acids (26). In this study in healthy volunteers, no effects of metformin on triglycerides and cholesterol were observed. The improvement of lipid metabolism by metformin may be a long-term effect. Therefore, short-term administration of metformin (e.g., two doses in two days in this study) may not cause a detectable effect. Alternatively, a notable effect in healthy people who have a normal regulation of plasma lipid levels may not be detected.

In conclusion, OCT1 polymorphisms have a significant effect on the pharmacokinetics of metformin in humans. The effect of OCT1 genotype on the pharmacokinetics of OCT1 substrates that are metabolized in the liver is likely to be clinically significant and warrants further study. Importantly, the pharmacodynamics of metformin is significantly affected by OCT1 genotype (activity) in mice and in healthy

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CHAPTER 5

EFFECT OF THE ORGANIC CATION TRANSPORTER 1, OCT1, ON HEPATIC TRIGLYCERIDE HOMEOSTASIS

Introduction

The organic cation transporter 1 (OCT1), encoded by *SLC22A1*, is a member of the Solute Carrier (SLC) Superfamily, which consists of more than 360 members from 45 subfamilies (HUGO Gene Nomenclature Committee). Many SLC members, including glucose transporters, amino acid transporters, and metal ion transporters, are important in maintaining cellular and total body homeostasis. The first OCT1, rat Oct1 (rOct1), was cloned in 1994 (1), followed by homology cloning of the human OCT1 isoform in 1997 from this laboratory and that of Koepsell (2, 3). The molecular identification of OCT1 has led to detailed characterization of its transport mechanism, substrate and inhibitor specificity, and tissue distribution, and importantly to the understanding of its role in drug disposition and response. However, the physiological role of OCT1 remains unknown.

It is now well known that the transport of organic cations by OCT1 is saturable and sensitive to membrane potential (4). OCT1 is considered an electrogenic facilitative transporter driven by the chemical gradient and intracellular negative potential. OCT1 can also act as an electroneutral organic cation exchanger. A variety of structurally diverse organic cations have been characterized as OCT1 substrates and/or inhibitors (5). These include clinically used drugs such as the anti-cancer agent oxaliplatin (6), the anti-diabetic agent metformin (7, 8) and the anti-hypertensive drug pindolol (9). OCT1 has therefore been proposed to play an important role in drug disposition and response. For example, we have demonstrated that OCT1 is required for the therapeutic effects of

metfomrin, and that OCT1 function affects metformin pharmacokinetics (Chapters 3 and 4).

Early studies showed that OCT1 has a wide tissue distribution with primary expression in the liver and lower levels in other tissues, including kidney, intestine, muscle and heart (2). Extensive microarray analysis reveals that in comparison to about 250 transporters analyzed in the SLC superfamily, OCT1 is the most abundantly expressed transporter in the human liver (10, 11). Moreover, microarray data from human tissues and cell lines indicate that OCT1 is expressed only at a low level (background or near background in the comparison) in all tissues except the liver (10, 11). The expression data strongly suggest that OCT1 has a critical function in the liver.

Because of its polyspecificity for many xenobiotics and its localization to the liver, it has been proposed that the primary role of OCT1 is to work in concert with drug-metabolizing enzymes (e.g., cytochrome P450s) in detoxification pathways in the liver. However, it is also possible that OCT1 has another primary function(s) in the body. For example, endogenous monoamines such as dopamine, norepinephrine, epinephrine, acetylcholine and histamine have been characterized as OCT1 substrates (12, 13). However, because of the low affinity of many of these compounds for OCT1, they may not be the physiologic substrates. It has been reported by Schinkel and coworkers that *Oct1* knockout mice appear to be normal with no obvious phenotypic abnormalities (14). In addition, we and others have identified a number of non-synonymous polymorphisms deficient in OCT1 function in healthy volunteers (15-17). Thus, a dozen years have passed since OCT1 was first cloned and yet we still have not answered the fundamental question of the physiological role of OCT1.

It is noteworthy, however, that many subtle but aberrant phenotypes only become amplified under specific extreme conditions. This seems to be the case for OCT1. In an attempt to study the role of OCT1 in metformin therapy of fatty liver disease, we generated *Oct1* knockout (*Oct1-/-*) and leptin deficient (*ob/ob*) double mutant mice (*Oct1-/-, ob/ob*). Surprisingly, the double mutant mice had markedly reduced fatty liver in comparison to *Oct1+/+*, *ob/ob* mice which are disease models of fatty liver, insulin resistance and diabetes (18, 19). The present study represents the preliminary characterization of the role of OCT1 in lipid and glucose metabolism and in the pathogenesis of fatty liver.

Materials and Methods

Animals. *Oct1-/-* mice were generated as described elsewhere (see Chapter 3). We initially planned to generate *Oct1-/-* mice in FVB/N inbred background to study the role of Oct1 in drug disposition and response. At the initiation of the present study, the original chimeras had been backcrossed with inbred FVB/N mice for five generations (F5 FVB/N). Therefore, *Oct1-/-* mice and the control *Oct1+/+* mice had, on average, a mixed genetic background of 97% FVB/N, 1.5% 129/OLA and 1.5% C57BL/6. However, because FVB mice are resistant to diet-induced obesity and are poor models to study metabolic syndrome and hepatic steatosis (20, 21), we started to backcross Oct1-/- mice with C57BL/6 inbred mice. C57BL/6 mice have been extensively used in studying metabolic syndrome and hepatic steatosis. The wild-type inbred C57BL/6J mice were purchased from Jackson laboratory (Bar Harbor, Maine).

Leptin deficient mice (*ob/ob*) have been used as a primary mouse model of hepatic steatosis (18, 19). To study the role of Oct1 in pathogenesis of fatty liver disease, we generated *Oct1* knockout (*Oct1-/-*), leptin deficient (*ob/ob*) double mutant (*Oct1-/-, ob/ob*) mice and the corresponding control mice (*Oct1+/+, ob/ob*) by intercrossing. In brief, our *Oct1-/-* mice (97% FVB/N background as described above) were mated with the heterozygous *OB/ob* mice (C57BL/6J background purchased from Jackson Laboratory) to obtain the double heterozygous mice (F2, *Oct1+/-, OB/ob*). The male *Oct1+/-, OB/ob* were then mated with the female *Oct1+/-, OB/ob* to obtain the required genotypes of mice.

In this preliminary characterization study, most of our animal studies were conducted in the F2 mice with mixed C57BL/6J (50%) and FVB/N (ca. 50%) backgrounds. We also performed some experiments with the F5 FVB/N mice. As the backcross was ongoing, pilot studies were also conducted on the mice with purer backgrounds of FVB/N or C57BL/6J. All animals were housed in a virus-free, temperature-controlled facility on a 12-hour light-dark cycle. They were free to access a standard diet of mouse food and water. All experiments on mice were approved by the Institutional Animal Care and Use Committee of the University of California at San Francisco.

Mouse genotyping. Genotypes were determined from tail tip or finger DNA by PCR followed by gel analysis or melt temperature analysis. The sense and anti-sense primers for mouse *Oct1* were 5'- ATG GGG TCT AAG GAG CCT GT-3' and 5'- CCG CTT GAG TGG TTC TCT TC -3', and the size of the PCR product was 413 bp. The

sense and anti-sense primers for the neomycin resistance gene (*Neo*) in the *Oct1* locus in *Oct1-/-* mice were 5'- CCG GCC GCT TGG GTG GAG AGG -3' and 5'- TCG GCA GGA GCA AGG TGA GAT GAC A -3', and the size of the PCR product was 299 bp. The sense and anti-sense primers for the mouse *leptin* gene were 5'- TGT CCA AGA TGG ACC AGA CTC -3' and 5'- ACT GGT CTG AGG CAG GGA GCA -3' as described in the genotyping protocol from Jackson Laboratory, and the size of PCR product was 155 bp. *Oct1* genotypes were directly determined from the PCR product sizes of *Oct1* and *Neo* in the agarose gel. The leptin deficiency in *ob/ob* mice is due to a single nucleotide mutation (Arg105Stop). To determine *leptin* genotypes, the PCR product was digested with the restriction enzyme *DdeI*. The melting temperature (at which the double strand DNA become single strands) of digested product was analyzed with a melting curve SNP (McSNP) analysis machine (Thermo Electron Co., MA). The wild type *leptin* is not cut by *DdeI*, and the PCR product with the mutation is cut into two fragments of 55 bp and 100 bp. DNA fragments of different sizes have different melting temperatures.

Physical characteristics of mice. Body weight (in grams) was measured for all of the mice. For growth curves, mice were weighed weekly from the week of weaning up to 22 weeks. Food consumption was monitored weekly for subgroups of mice, and the average daily food intake was calculated for each mouse. Subgroups were sacrificed and dissected to obtain liver weights and weights of individual adipose pads (epididymal or gonadal, and peri-kidney).

Histological analysis. Mice were anesthetized with a mixture of ketamine and medetomidine and sacrificed by heart puncture. Wedges of liver and adipose tissues from epididymal fat pads were removed quickly and fixed overnight in neutral phosphate-buffered 10% formaldehyde solution. The tissues were embedded in paraffin, cut into thin sections and mounted on glass slides. The tissue sections were stained with hematoxylin and eosin to demonstrate general hepatic and adipose histology.

Plasma activities of liver-associated enzymes. Mice were anesthetized with a mixture of ketamine and medetomidine, and blood samples were collected by heart puncture. Plasma was separated from the blood and stored at -20°C until analysis. Alanine aminotransferase (ALT), aspartate aminotransferasae (AST) and alkaline phosphatase (ALKP) activities were measured in plasma by the Clinical Laboratory of the San Francisco General Hospital.

Liver triglyceride quantitation. From each mouse, 60 to 100 mg of liver was homogenized in 4 ml of chloroform-methanol (2:1). A total of 0.8 ml of 50 mM NaCl was then added to each sample. Samples were then centrifuged and the lower organic phase was removed. An aliquot of the organic phase was collected, dried under nitrogen gas, and resuspended in 2% Triton X-100. Triglycerides were assayed by the colorimetric method using an enzymatic measurement kit (Sigma).

Plasma glucose, insulin, and triglycerides. Blood samples were collected by tail bleeding into heparinized micro-hematocrit capillary tubes (Fisher, Pittsburgh, PA).

Plasma was isolated by centrifuging the capillary tubes in microhematocrit centrifuge (Thermo Electron Corporation, MA). Plasma glucose was determined using the glucose oxidase assay kit (Sigma). Plasma insulin was determined using an insulin EIA kit (Alpco Diagnostics, Salem, NH). Plasma triglyceride was measured using the enzymatic measurement kit from Sigma.

VLDL production assay. Mice were fasted for five hours before injection with tyloxapol (Triton-1339, Sigma) as described before (22, 23). Mice were injected *via* the tail vein with 500 mg/kg tyloxapol dissolved in saline at a concentration of 0.15 g/ml. Tail bleeds were done just before injection (0 min) and 30, 60, 90 and 120 min following injection. Plasma triglycerides were assayed as described above. The slope of the line of triglyceride concentration *versus* time denotes the production rate of very low density lipoproteins (VLDL).

Triglyceride quantitation in primary mouse hepatocytes. Primary hepatocytes were isolated from wild-type and *Oct1-/-* mice in FVB/N background by the UCSF Liver Center using the standard collagenase method (24). The cells were plated in William's E medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1% bovine albumin, 0.1 μM dexamethasone, 2 mM L-glutamine, 1X ITS (100X Insulin-Transferrin-Selenium from Invitrogen) at a density of 1.5 x 10^5 cells/cm^2 on collagen-coated 6-well plates (Becton Dickenson, Bedford, MA). After attachment (2 – 3 h), hepatocytes were maintained in the completed medium with 0.25 mg/ml matrigel (BD

Sciences, San Jose, CA) for 16 hours. After another 24-hour culture in regular medium, triglycerides were assayed as described above.

Immunoblots. Liver samples were homogenized in ice-cold lysis buffer using a tissue homogenizer at 4°C. The lysis buffer consisted of 20 mM Tris pH 7.4, 1% Triton-X 100, 150 mM NaCl, 250 mM sucrose, 50 mM NaF, 2.5 mM $Na_3P_2O_4$, 2 mM DTT and 10 mM Na_3VO_4 with the protease inhibitors dissolved from a Complete® protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN). After centrifugation for 20 minutes at 14,000 g at 4°C, the supernatants were removed for determination of protein content and separated on 10% SDS-PAGE gels. Forty microgram (μg) of proteins were separated from the supernatant and transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C with Tris-buffered saline with 0.05% Tween 20 and 5% nonfat milk. Immunoblotting was performed following standard procedures and the signals were detected by chemiluminescence reagents (Amersham). Primary antibodies were directed against AMPKα phosphorylated at Thr172 and β-actin (Cell Signaling Technology, Danvers, MA).

***hOCT1*-transgenic *C. elegans* and fat staining.** Human *OCT1* (*hOCT1*) cDNA in pcDNA3 plasmid was cut with *SpeI* and *NotI*, and was inserted into the plasmid vector pJM16 (kindly provided by Dr. Ashrafi at University of California at San Francisco), which has a worm intestine-specific promoter, ges-1p, to generate the construction of *ges::hOCT1*. An *XbaI* site was introduced into the *ges::hOCT1* and the pPD95-79 vector (kindly provided by Dr. Ashrafi) respectively. The poly-adenosine tail (unc-54) in the

pPD95-79 was released by cutting the vector with *XbaI* and *EagI*, and then inserted into the *ges::hOCT1* which was cut by *XbaI* and *NotI*. The construct of hOCT1 driven by the ges-1p promoter and followed by unc-54 (*ges::hOCT1::unc54*) in pJM16 was confirmed by sequencing. To generate *hOCT1*-expressing animals, the *ges::hOCT1::unc54* released from the pJM16 by *KpnI* and *PvuI* digestion was injected as described (25) at 50 ng/µl into animals to generate independent transgenic lines. The coinjection marker (rfp in pJM16) was also injected to facilitate selection of positive animals. hOCT1-expressing animals were confirmed by PCR with specific primers.

The fat staining with Nile Red (5H-benzo[α]phenoxazine-5-one, 9-diethylamino) was described (26). In brief, the Nile Red powder (N-1142, Molecular Probes, Invitrogen, CA) was dissolved in acetone at 500 µg/ml, diluted in 1X phosphate buffered saline (PBS) and added on top of nematode growth media plates already seeded with OP50 bacteria, to a final concentration of 0.05 µg/ml. Worms were placed on these plates as starved L1s. Their staining phenotypes were assessed prior to starvation at the L4 and the young adult stages. Fat content was monitored by fluorescence microscopy.

Glucose uptake in *Xenopus laevis* oocytes and mammalian cell lines. The expression of human OCT1 in *Xenopus laevis* oocytes was described in Chapter 2. The mammalian cell lines, HEK-OCT1 cells and the mock cells transfected with the empty vector, were generated and cultured as described in Chapter 3. Glucose uptakes in oocytes and in the mammalian cells were conducted in a manner similar to MPP^+ uptakes in oocytes as described in Chapter 2 and in mammalian cells as described in Chapter 3, respectively. The uptake substrate consisted of 3-O-methyl-D-[1-^{3}H]glucose (^{3}H-3-OMG,

4.0 Ci/mmol, GE Healthcare Bio-Sciences, Piscataway, NJ) plus unlabeled 3-OMG (Sigma). The time of uptake ranged from 2 minutes to 4 hours.

Fatty acid uptake in HEK-OCT1 and mock cells. Oleic acid uptakes in HEK-OCT1 and mock cells were conducted in a manner similar to MPP^+ uptakes in mammalian cells as described in Chapter 3. In brief, uptake of ^{3}H-oleic acid was performed by incubating the cells grown on poly-D-lysine coated 24-well plates with uptake buffer (40 μM sodium oleate, 10 μM fatty acid free bovine serum albumin, 5 μCi/ml ^{3}H-oleic acid in PBS with 1 mM $MgCl_2$, 1.2 mM $CaCl_2$). The uptake was conducted at 37° for 10 minutes and stopped by addition of ice cold stop buffer. The cells were washed three times with ice-cold PBS, and then lysed with 0.1 N NaOH and 0.1% SDS. The lysate was used for scintillation counting (Beckman Instruments, Palo Alto, CA) and for the BCA protein assay (Pierce, Rockford, IL).

Statistical analysis. Unless indicated, the data are presented as mean ± standard error (SE) or standard deviation (SD) from a representative experiment. In general the experiments were repeated at least twice. Two tailed *Student's* t-test was used to analyze data, when appropriate. For multiple comparison tests, *ANOVA* was used following a *Dunnett's* test. A P value of < 0.05 was considered statistically significant.

Results

Hepatic triglyceride accumulation was much less in *Oct1-/-, ob/ob* mice than in *Oct1+/+, ob/ob* mice. The growth curve was similar between our *Oct1-/-, ob/ob* mice and *Oct1+/+, ob/ob* mice both of which had a genetic background of 50% FVB/N and 50% C57BL/6J (data not shown). At the age of 20 weeks, no difference in body weight was observed between the two strains (Figure 5.1A, Table 5.1). The original leptin-deficient *ob/ob* mice have been extensively used as the disease models to study fatty liver, insulin resistance and diabetes. However, we found that at the age of 20 weeks, the *Oct1-/-, ob/ob* mice had a significantly smaller liver size and a smaller ratio of liver to body weight, as compared with the *Oct1+/+, ob/ob* mice (Figure 5.1B, Table 5.1). The livers from the *Oct1+/+, ob/ob* mice appeared to be paler. Subsequent histology analysis revealed little fat accumulation in the female *Oct1-/-, ob/ob* mice, while the female *Oct1+/+, ob/ob* mice developed severe fatty liver (Figure 5.1C). The hepatic fat accumulation in male *Oct1-/-, ob/ob* mice was also much milder than that in male *Oct1+/+, ob/ob* mice (data not shown). The differences in hepatic fat content were more pronounced in females than in males. We measured triglyceride content in the livers of female mice. The hepatic triglyceride content was significantly less in the female *Oct1-/-, ob/ob* mice than in the female *Oct1+/+, ob/ob* mice (Figure 5.2). These data strongly suggest that *Oct1* deficiency protects leptin-deficient *ob/ob* mice from developing severe fatty liver, and that Oct1 may play a role in triglyceride accumulation in the liver.

***Oct1* deficiency improved liver injury in *ob/ob* mice.** Leptin-deficient *ob/ob* mice not only severely accumulate fat in the liver, but also cause liver injury (19). We

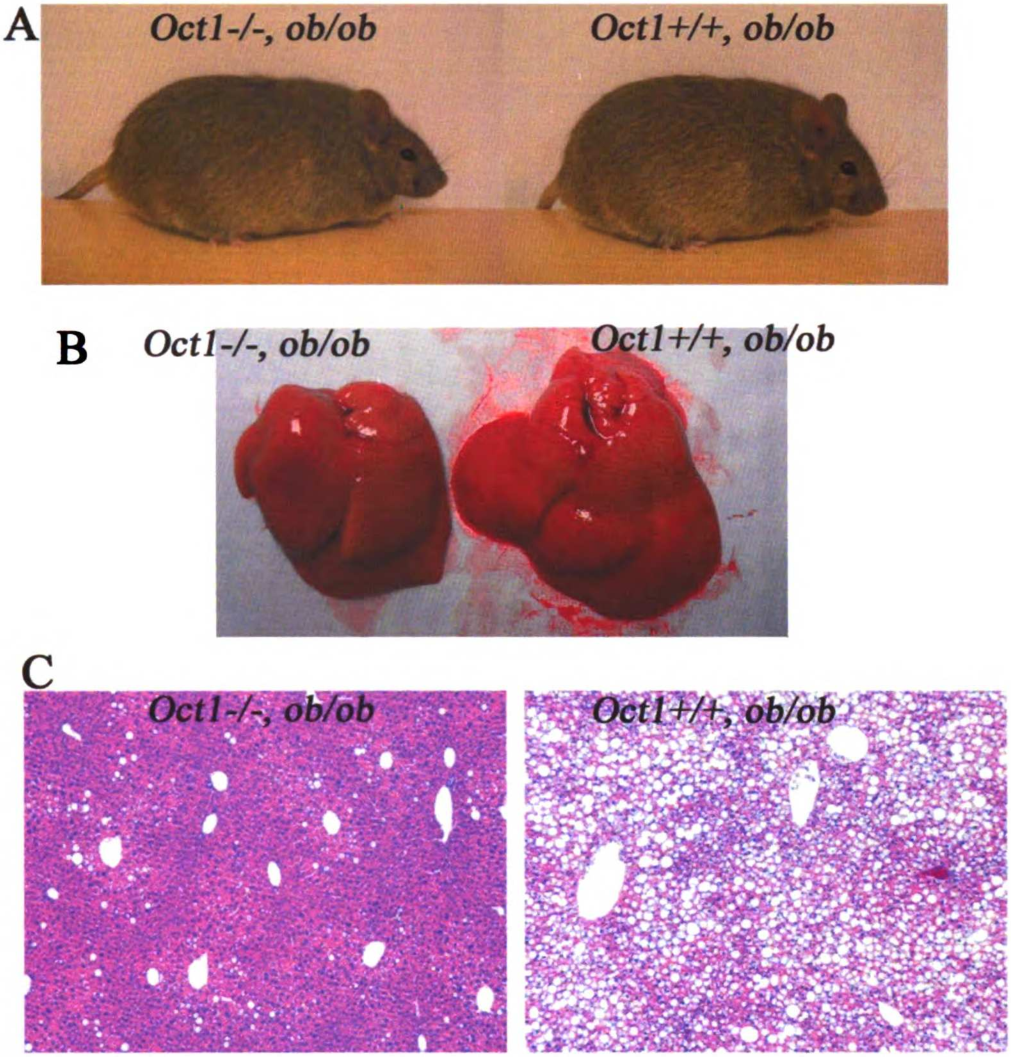


Figure 5.1. Representative body (A), liver (B), and hepatic histology (C) for one female *Oct1-/-, ob/ob* and one female *Oct1+/+, ob/ob* mouse. Deletion of *Oct1* resulted in a reduced liver size, but not body size in these mice. Liver to body weight ratio was reduced in *Oct1-/-, ob/ob* mice. For hepatic histology, formalin-fixed, paraffin-embedded, hematoxylin and eosin-stained sections were photographed and digital imagines were captured using a SpotCamera. The magnification is 50×. Liver slices from *Oct1-/-, ob/ob* mice had markedly less fat than *Oct1+/+, ob/ob* mice.

Table 5.1. Effect of *Oct1* deletion on the liver size of leptin deficient (*ob/ob*) mice.

Gender	Genotype	Body Weight (g)	Liver Weight (g)	Liver/Body Weight ratio (%)
Female	*Oct1-/-, ob/ob (n = 9)*	56.2 ± 5.5	2.41 ± 0.52	4.33 ± 1.11
	Oct1+/+, ob/ob (n = 12)	52.0 ± 4.8	3.85 ± 0.51*	7.48 ± 1.25*
Male	*Oct1-/-, ob/ob* (n = 10)	54.9 ± 6.6	3.34 ± 0.68	6.11 ± 1.18
	Oct1+/+, ob/ob (n = 8)	55.1 ± 5.5	5.02 ± 1.06*	9.13 ± 1.68*

Note: The mice were fasted for 16 hours before sacrifice.

*$P < 0.001$ *versus Oct1-/-, ob/ob* of the same gender. Data are presented as mean ± SD.

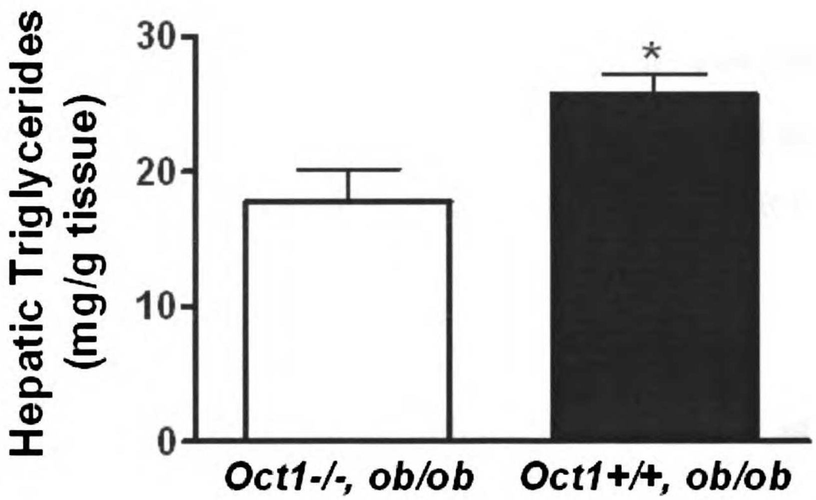
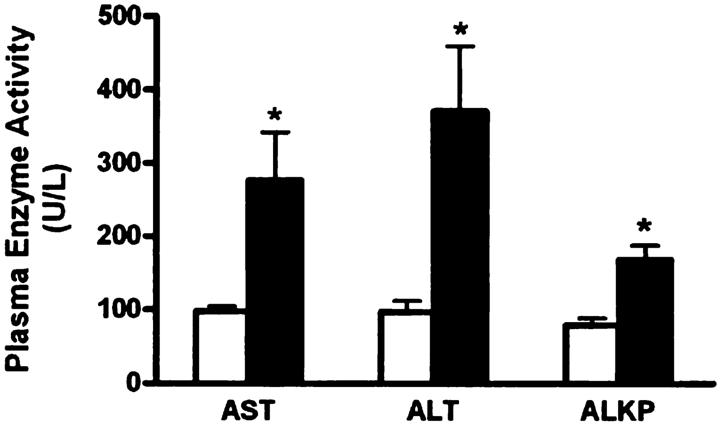


Figure 5.2. Hepatic triglyceride content in the livers of *Oct1-/-, ob/ob* (n = 9) and *Oct1+/+, ob/ob* (n = 11) female mice. The mice were sacrificed at 20 weeks. The liver samples were homogenized in chlorform and methanol (2:1). NaCl (50mM) was then mixed with the homogenate. The lower phase was removed, and evaporated under nitrogen. The triglyceride concentration in the reconstitution was determined by using an enzymatic measurement kit (Sigma) by the colorimetric method. *$P = 0.01$ *versus Oct1-/-, ob/ob* mice. The data are presented as mean ± SE.

measured the activities of three liver-associated enzymes: ALT, AST and AK in the plasma from 20-week old *Oct1-/-, ob/ob* mice and *Oct1+/+, ob/ob* mice. As shown in Figure 5.3, *Oct1-/-, ob/ob* mice had markedly reduced enzyme activities for all three enzymes tested when compared with *Oct1+/+, ob/ob* mice, suggesting that liver injury was improved by *Oct1* deficiency. The improvement was more dramatic in females than in males: the enzyme activities for *Oct1-/-, ob/ob* female mice were comparable to those for lean mice (data not shown). The results of the liver-associated enzyme tests were consistent with the above observations that *Oct1* deficiency caused less fatty liver in *ob/ob* mice.

***Oct1* deficiency was associated with increased peripheral adiposity in *ob/ob* mice.** Enhanced hepatic triglyceride accumulation is usually accompanied by increased total body adiposity. We measured peripheral adiposity by weighing epididymal (for male mice) or gonadal (for female mice) and peri-kidney fat pads. Unexpectedly, 20-week-old *Oct1-/-, ob/ob* mice had a significantly bigger size of epididymal or gonadal fat pad than did *Oct1+/+, ob/ob* mice (Figure 5.4A, Table 5.2), contrasting with the opposite difference in liver size between the two strains. The male *Oct1-/-, ob/ob* mice also had significantly larger peri-kidney fat pads compared with those of the male *Oct1+/+, ob/ob* mice. *Oct1-/-, ob/ob* mice of younger ages also tended to have more epididymal/gonadal fat deposition than that of *Oct1+/+, ob/ob* mice of same ages, but the differences did not reach statistical significance except for 16-week-old male mice. Interestingly, the sizes of the peripheral fat pads that were measured decreased as the age increased for both strains, suggesting age-dependent degeneration of peripheral fat tissues. In histological analysis,

A: Female mice



B: Male mice

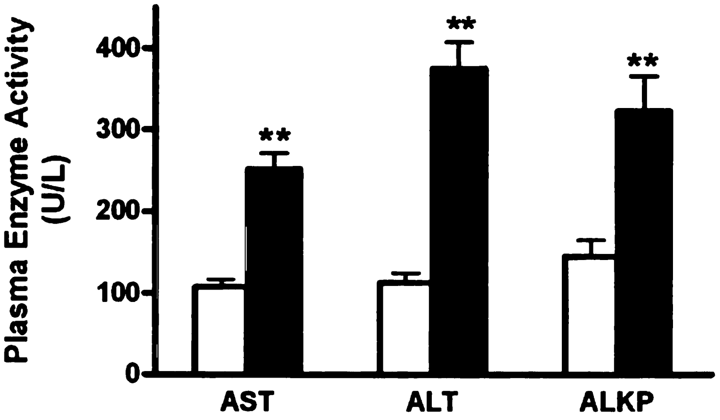


Figure 5.3. The plasma activities of three liver-associated enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALKP), in 20-week-old *Oct1-/-, ob/ob* (white bars) and *Oct1+/+, ob/ob* (black bars) mice. **A:** females, 9 *Oct1-/-, ob/ob* and 11 *Oct1+/+, ob/ob* mice; **B:** males, 10 *Oct1-/-, ob/ob* and 7 *Oct1+/+, ob/ob* mice. Mice were fasted for 16 hours before blood collection. *$P < 0.05$, **$P < 0.001$ *versus Oct1-/-, ob/ob*. The data are presented as mean ± SE.

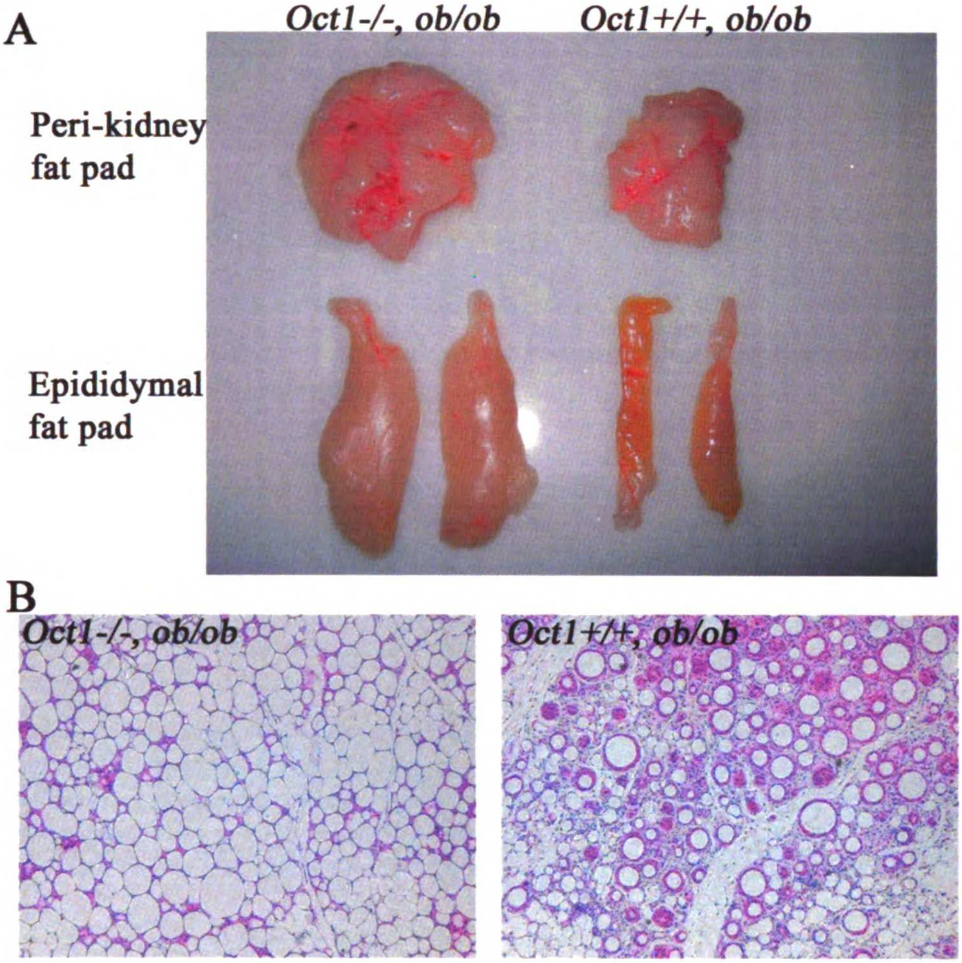


Figure 5.4. Representative peri-kidney fat pad (A, upper), epididymal fat pad (A, lower), and epididymal fat histology (B) for one male *Oct1-/-, ob/ob* and one male *Oct1+/+, ob/ob* mouse. The two 20-week-old mice had a similar body weight (*Oct1-/-, ob/ob*, 59.5 g; *Oct1+/+, ob/ob*, 56.0 g). Deletion of *Oct1* resulted in a greater size for the fat pads indicated by increased number and size of adipocytes. For epididymal fat histology, formalin-fixed, paraffin-embedded, hematoxylin and eosin-stained sections were photographed and digital images were captured using a SpotCamera. The magnification is 50×. The slices from *Oct1-/-, ob/ob* mice had more lipid droplets, and those from *Oct1+/+, ob/ob* mice had apparent fibrous tissues.

Table 5.2. Peripheral adiposity (epididymal/gonadal and peri-kidney fat pads) in different ages of leptin deficient mice (*ob/ob*) with different *Oct1* genotypes (*Oct1-/-* and *Oct1+/+*).

Age (wk)	Gender	Genotype	*BW (g)	*Epi/Gona (g)	**Peri-Kid (g)
16	Female	*Oct1-/-, ob/ob* (n = 6)	54.9 ± 10.5	4.07 ± 0.79	2.72 ± 0.70
		Oct1+/+, ob/ob (n = 4)	49.6 ± 5.0	3.84 ± 0.89	2.32 ± 0.43
	Male	*Oct1-/-, ob/ob* (n = 6)	52.6 ± 8.6	2.41 ± 0.90	3.39 ± 0.96
		Oct1+/+, ob/ob (n = 4)	55.7 ± 4.4	1.62 ± 0.89[a]	2.71 ± 0.52[a]
20	Female	*Oct1-/-, ob/ob* (n = *9)*	56.2 ± 5.5	3.95 ± 0.72	2.38 ± 0.65
		Oct1+/+, ob/ob (n = 12)	52.0 ± 4.8	3.22 ± 0.73[a]	2.31 ± 0.44
	Male	*Oct1-/-, ob/ob* (n = 10)	54.9 ± 6.6	1.64 ± 0.69[b]	3.05 ± 0.82
		Oct1+/+, ob/ob (n = 8)	55.1 ± 5.5	0.74 ± 0.28[a,b]	2.29 ± 0.76[a]

*BW, Body weight; Epi, Epididymal fat pad; Gona, Gonadal fat pad. ** Peri-Kid, Peri-kidney fat pad.

[a] $P < 0.05$ *versus Oct1-/-, ob/ob* of the same gender and age. [b] $P < 0.05$ *versus* 16-week-old mice of the same genotype and gender. Data are presented as mean ± SD.

the epididymal adipose tissue depot isolated from the 20-week-old *Oct1-/-, ob/ob* mice had significantly more and larger adipocytes, when compared with the *Oct1+/+, ob/ob* mice (Figure 5.4B). The fibrous tissue was obvious among the epididymal adipose tissue isolated from the *Oct1+/+, ob/ob* mice. These data suggest that the increased triglyceride accumulation in the liver may not result from an overall body adiposity increase for *Oct1+/+, ob/ob* mice *versus Oct1-/-, ob/ob* mice.

Plasma glucose, insulin, triglyceride, and cholesterol levels were different between *Oct1-/-, ob/ob* and *Oct1+/+, ob/ob* mice. The observed differences in hepatic triglyceride accumulation, liver injury and peripheral adiposity suggested different metabolism of nutrient substances between the two strains. We measured several plasma metabolic indices in the 20-week-old mice of the two genotypes of both genders (Table 5.3). When subjected to an overnight fast (16-hour), significant gender-dependent differences in plasma levels of glucose, insulin, triglyceride, and/or cholesterol were found between *Oct1-/-, ob/ob* and *Oct1+/+, ob/ob* mice. Male *Oct1-/-, ob/ob* mice had significantly higher glucose but lower insulin and cholesterol levels in the plasma, and female *Oct1-/-, ob/ob* mice had significantly lower insulin and triglyceride levels, when compared with respective *Oct1+/+, ob/ob* mice of same gender. The metabolic indices measured in the *ob/ob* mice of both *Oct1* genotypes were generally higher than those in lean mice (data not shown), indicating metabolic abnormalities in leptin-deficient obese mice regardless of *Oct1* genotypes. Collectively, the data suggest that Oct1 plays a role in lipid and glucose homeostasis, but the specific role is affected by gender. Lower insulin

Table 5.3. Effect of *Oct1* deletion on insulin, triglyceride, cholesterol and glucose levels in the plasma in leptin deficient mice (*ob/ob*) mice.

Gender	Genotype	Insulin (ng/ml)	Triglyceride (mg/dL)	Cholesterol (mg/dL)	Glucose (mg/dL)
Female	*Oct1-/-, ob/ob*	2.59 ± 1.28	88 ± 32	184 ± 56	165 ± 25
	Oct1+/+, ob/ob	3.46 ± 1.40*	154 ± 70*	220 ± 27	203 ± 87
Male	*Oct1-/-, ob/ob*	2.31 ± 0.62	157 ± 106	191 ± 37	397 ± 103
	Oct1+/+, ob/ob	4.06 ± 1.47*	166 ± 40	253 ± 33*	258 ± 103*

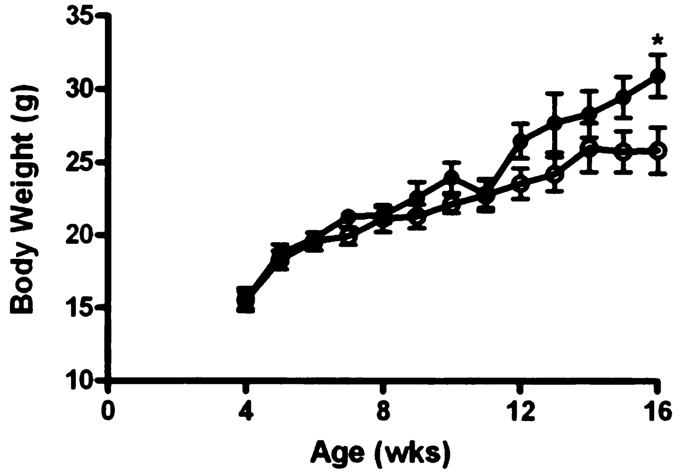
Note: Blood samples were collected from the mice subjected to 16 hours overnight fasting.

*$P < 0.05$ *versus Oct1-/-, ob/ob* of the same gender. 4-12 mice were used for each group. Data are presented as mean ± SD.

levels were observed in Oct1-/-, ob/ob mice irrespective of gender, suggesting that Oct1 deficiency may cause less insulin secretion.

***Oct1* deficiency resulted in subtle but significant phonotypic changes related to nutrient metabolism in lean mice.** To remove the confounding effects of leptin deficiency, we performed studies to examine lean *Oct1-/-* and *Oct1+/+* mice in the absence of leptin deficiency. First, we used the mice that had been backcrossed with FVB/N inbred mice for six generations. No difference in body weight was found between *Oct1-/-* and *Oct1+/+* mice up to two months of age. However, the growth curves began to diverge at about 9 – 12 weeks after birth (Figure 5.5). When the mice were sacrificed at 16 weeks of age, both male and female *Oct1-/-* mice had significantly lower body weights than those of the corresponding *Oct1+/+* littermates. The livers of *Oct1-/-* mice weighed slightly but significantly less than those of *Oct+/+* mice (Table 5.4). The liver to body weight ratios were also significantly decreased in the female *Oct1-/-* mice. Consistent with the smaller liver weights, triglyceride content was reduced in the female *Oct1-/-* mice (Figure 5.6). Hepatocytes isolated from *Oct1-/-* mice had significantly lower triglyceride content compared with those isolated from *Oct1+/+* mice (Figure 5.7). However, when subjected to an overnight fast (16 hours), *Oct1-/-* mice had significantly higher glucose levels in the plasma than *Oct+/+* mice (Figure 5.8). There were no differences in plasma insulin, triglyceride and cholesterol levels between the lean *Oct1-/-* and *Oct1+/+* mice (data not shown). Both genotypes of mice showed similar, normal hepatic histology (data not shown).

A: Female mice



B: Male mice

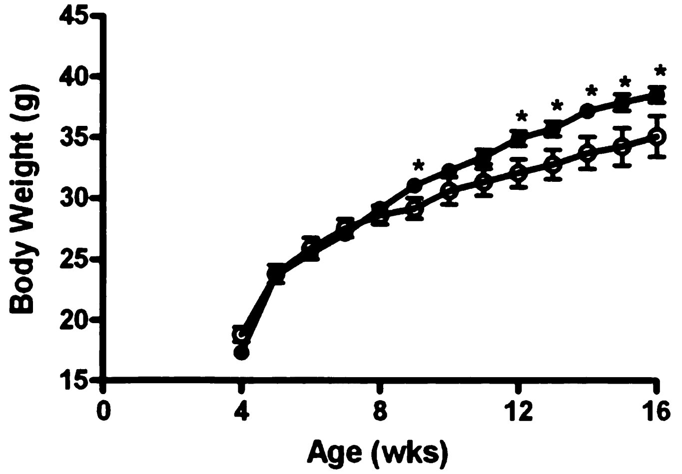


Figure 5.5. Growth curves of *Oct1-/-* (open circle) and *Oct1+/+* (solid circle) lean mice. Body weight monitoring was begun 1 week after weaning. The body weight was measured on each Monday. The mice had been backcrossed with FVB/N inbred mice for six generations. A: females; B: males. $*P < 0.05$ *versus Oct1-/-* of the same gender. 5-10 mice were used for each group. Data are presented as mean ± SE.

Table 5.4. Effect of *Oct1* deletion on the liver size of lean mice.

Gender	Genotype	Body Weight (g)	Liver Weight (g)	Liver/Body Weight ratio (%)
Female	*Oct1-/-* (n = 6)	25.4 ± 3.8	0.95 ± 0.13	3.75 ± 0.08
	Oct1+/+ (n = 5)	30.8 ± 3.8*	1.29 ± 0.17*	4.17 ± 0.10*
Male	*Oct1-/-* (n = 4)	32.6 ± 5.3	1.19 ± 0.16	3.65 ± 0.12
	Oct1+/+ (n = 8)	36.6 ± 2.2*	1.39 ± 0.11*	3.80 ± 0.33

Note: The mice had been backcrossed with FVB/N inbred mice for six generations. The mice were fasted for 16 hours before sacrifice.

*$P < 0.05$ *versus Oct1-/-* of the same gender. Data are presented as Mean ± SD.

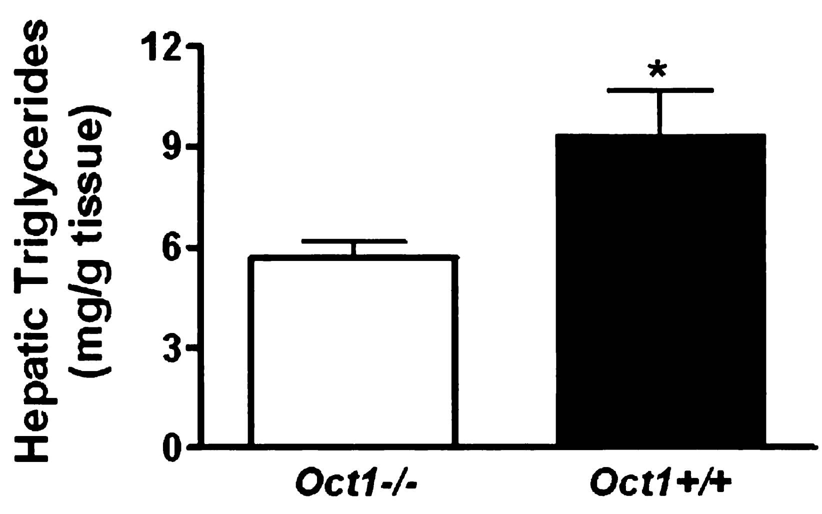


Figure 5.6. Hepatic triglyceride content in the livers of *Oct1-/-* (n = 5) and *Oct1+/+* (n = 5) female lean mice. The mice had been backcrossed with FVB/N inbred mice for six generations. The mice were sacrificed at 16 weeks old. The liver samples were prepared, and the triglycerides were measured as described in Figure 5.2. *$P = 0.03$ *versus Oct1-/-* mice. The data are presented as mean ± SE.

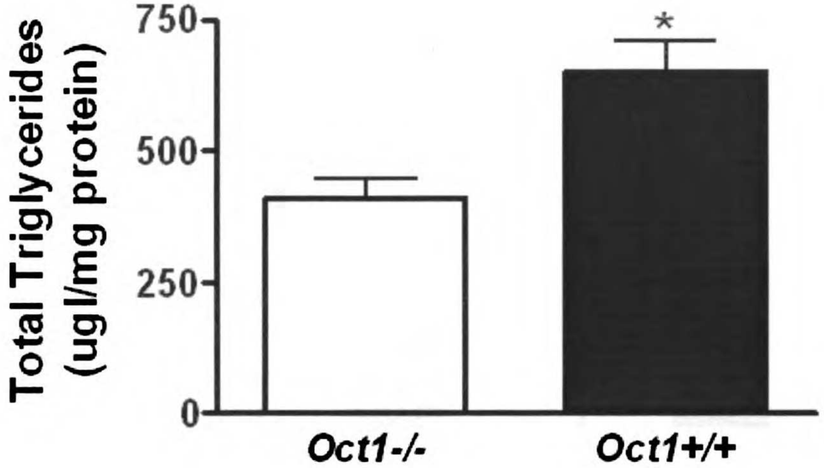


Figure 5.7. The levels of total triglycerides in primary hepatocytes isolated from *Oct1-/-* and *Oct1+/+* mice. The isolation and culture of primary hepatocytes are described in the Materials and Methods section. Triglycerides were extracted and measured. The figure represents triplicate measurements on primary hepatocytes prepared from one mouse of each genotype. The experiments were repeated three times, and similar results were obtained. *$P = 0.004$ *versus Oct1-/-* mice. The data are presented as mean ± SE.

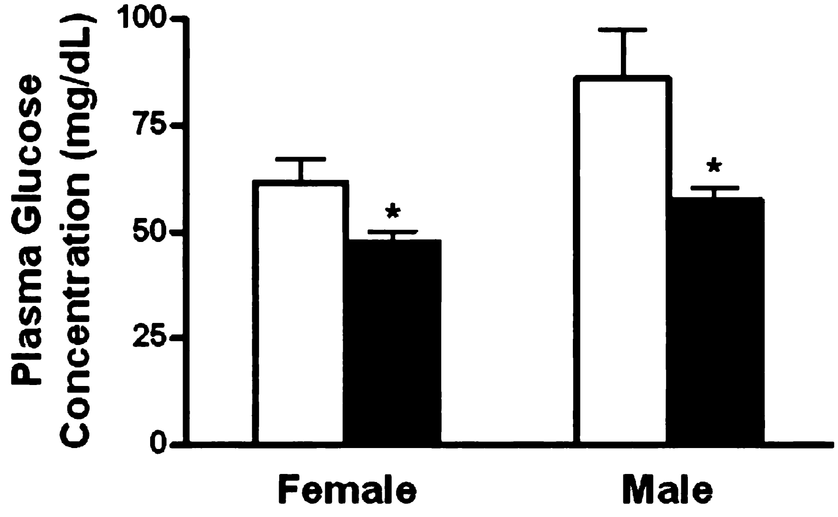


Figure 5.8. Fasting glucose levels in the plasma of *Oct1-/-* (white bars) and *Oct1+/+* (black bars) lean mice. The mice had been backcrossed with FVB/N inbred mice for six generations. The mice were fasted for 16 hours before blood collection. Between five and ten mice were used for each group. $*P < 0.05$ *versus Oct1-/-* mice. The data are presented as mean ± SE.

A pilot study was conducted using three *Oct1-/-* mice and three *Oct1+/+* mice (12 weeks old) that had been backcrossed with C57BL/6J for six generations. Body weight, liver weight and liver to body weight ratio were compared between the two groups of mice at 11 weeks of age. The body weight of *Oct1-/-* mice tended to be smaller (20.9 ± 2.2 g *vs.* 22.6 ± 0.47 g, n = 3 for each group), but the difference was not significant. However, both liver weight and liver to body weight ratio were significantly smaller in the *Oct1-/-* mice in comparison to the *Oct1+/+* mice (liver weight: 0.73 ± 0.02 g *versus* 0.95 ± 0.026 g, *P* = 0.0003; liver to body weight ratio: 3.51 ± 0.36 % *versus* 4.21 ± 0.11 %, *P* = 0.032). These data from healthy mice suggest that even in the absence of leptin deficiency or hepatic steatosis, Oct1 plays a role in hepatic triglyceride accumulation and glucose metabolism. However, the effects of Oct1 in healthy mice were attenuated in comparison to those in leptin-deficient obese mice.

Transgenic *C. elegans* over-expressing human OCT1 had increased fat accumulation in the intestine. The phenotypes from *Oct1* deletion in obese mice and healthy mice were reminiscent of previous studies in *C. elegans*. By using RNAi to an *OCT1* homolog in *C. elegans*, Ashrafi *et al.* demonstrated that knocking down the expression of the *OCT1* homolog caused a reduced fat phenotype (26). In the present study, we injected a DNA construct consisting of a worm intestinal-specific promoter and a human OCT1 cDNA sequence into *C. elegans*. Positive transgenic *C. elegans* over-expressing human OCT1 were selected and confirmed by a co-injected marker (rfp fluoresce) and RT-PCR. The transgenic worms and wild-type control worms were fed *Escherichia coli* supplemented with Nile red, a dye staining fat droplets. We found that

OCT1-transgenic worms deposited much more fat in the intestine, the major site accumulating fat in *C. elegans*, when compared with wild-type control worms (Figure 5.9). The data, in agreement with those from *Oct1-/-* mice, suggest that Oct1 has a physiologic role in triglyceride accumulation.

Female *Oct1-/-, ob/ob* mice had a lower rate of hepatic production/efflux of VLDL than female *Oct1+/+, ob/ob* mice. We measured triglyceride levels in the plasma of mice over time after dosing the mice with tyloxapol, a compound that prevents hydrolysis/degradation of VLDL in the plasma. As shown in Figure 5.10, the production/secretion rate of VLDL was lower in the female *Oct1-/-, ob/ob* mice in comparison to the rate in the female *Oct1+/+, ob/ob* mice (12.6 mg/dL/min in *Oct1-/-, ob/ob versus* 20.5 mg/dL/min in *Oct1+/+, ob/ob*, $P < 0.0001$). The production rates of VLDL were similar in male *ob/ob* mice regardless of *Oct1* genotypes. The reason for the gender differences was unknown. Nonetheless, the data indicate that *Oct1* deficiency does not increase VLDL production *per se* in mice and suggest that the reduced hepatic accumulation of triglycerides in *Oct1-/-* mice can not be explained by an increased output.

***Oct1* deficiency enhanced AMPK phosphorylation in the liver and increased food intake in mice.** AMP-activated protein kinase (AMPK) is an important enzyme that works as an intracellular "energy sensor" which becomes activated in situations of net energy consumption (27). We examined whether the reduced triglyceride accumulation in the liver of *Oct1-/-* mouse is related to AMPK activity. Ten-week-old F5

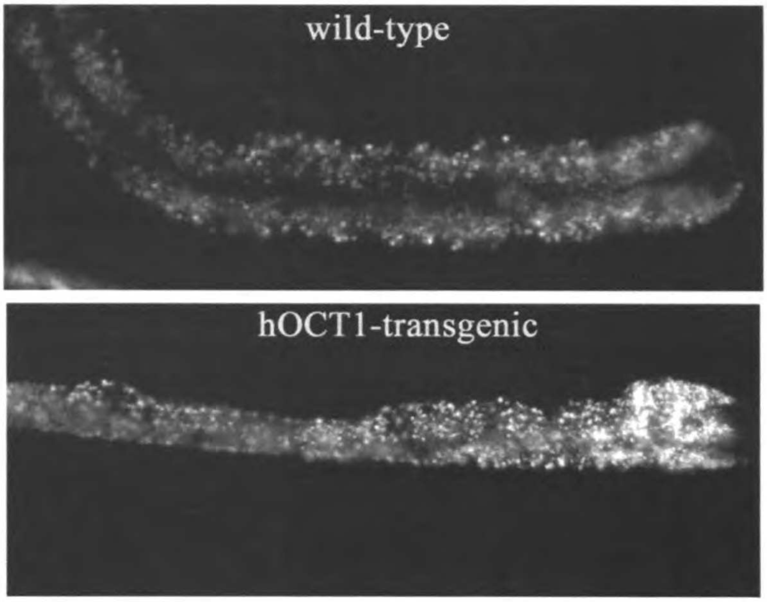


Figure 5.9. Fat accumulation in non-starved young adult wild-type and hOCT1-transgenic *C.elegans*. The transgenic worm was generated, and the worms were stained with Nile red as described in the Materials and Methods section. The magnification is 160×. In each image, anterior is positioned to the right.

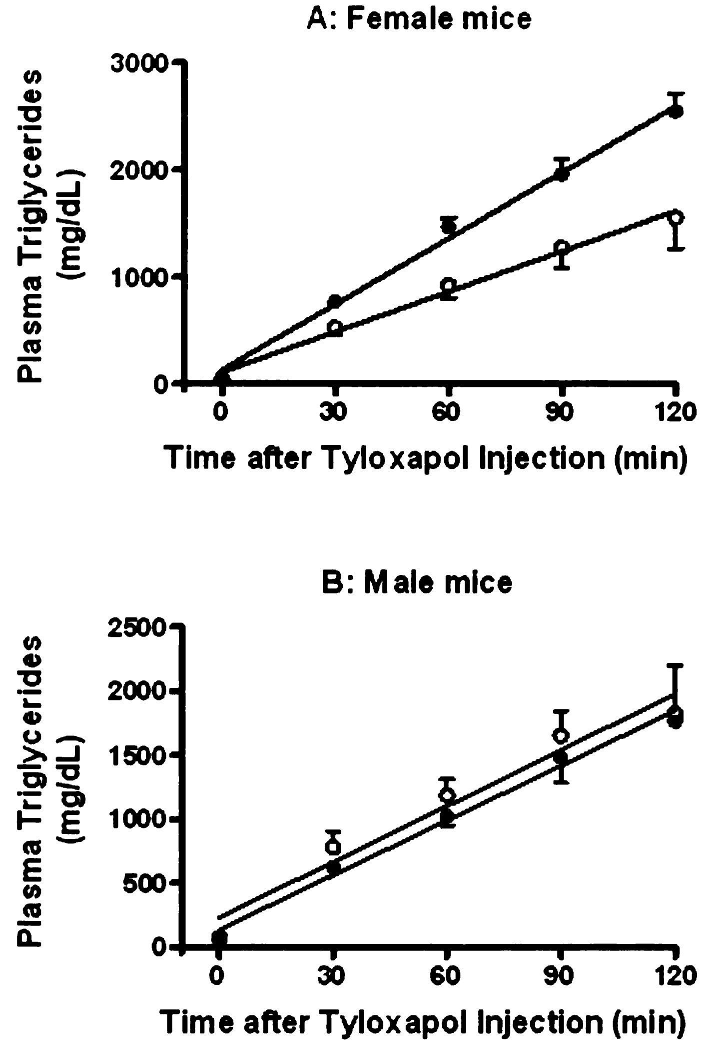


Figure 5.10. VLDL production in *Oct1-/-, ob/ob* (open circle) and *Oct1+/+, ob/ob* (solid circle) mice. Mice fasted for five hours were injected with 500 mg/kg tyloxapol *via* the tail vein. Plasma triglycerides were assayed at 0, 30, 60, 90 and 120 min as described in the Materials and Methods section. The slope of the line indicates the rate of VLDL production. A: females; B: males. Four mice were used for each group. The data are presented as mean ± SE.

FVB/N lean mice of both *Oct1* genotypes were sacrificed, and AMPK phosphorylation in the liver was measured. As shown in Figure 5.11A, AMPK phosphorylation in the livers of lean *Oct1-/-* mice was more apparent than in the livers of lean *Oct1+/+* mice. Further, we examined AMPK phosphorylation in the livers of leptin-deficient obese mice with the two *Oct1* genotypes. Hepatic AMPK phosphorylaton was substantially enhanced by *Oct1* deficiency in the obese mice (Figure 5.11B). In addition, we measured food intake every week for the two genotypes of F2 male lean mice (50% C57BL/6J and ca. 50% FVB/N) from five weeks to 16 weeks. On average, the male *Oct1-/-* mice ate approximately 8% more than did the male *Oct1+/+* mice (Figure 5.12A). Similar results of food intake difference (ca. 6%) were obtained between *Oct1-/-, ob/ob* and *Oct1+/+, ob/ob* mice (Figure 5.12B). Although further food intake measurements on individual mice and other measurements such as oxygen consumption may be more informative, these data collectively suggest a faster energy turnover in *Oct1-/-* mice, which may contribute to the lower triglyceride accumulation in the liver.

OCT1 was not a fatty acid or a glucose transporter. OCT1 has been well characterized as a drug transporter (4, 5). We examined whether Oct1 is also a fatty acid or glucose transporter. We performed ^{3}H-oleic acid uptake and 3-O-methyl-D-[1-^{3}H]glucose (^{3}H-3-OMG) uptake using different heterologous systems over-expressing human OCT1. No difference in ^{3}H-oleic acid uptake was found between HEK-OCT1 and mock transfected cells (10 minute uptake: 0.0014 ± 0.00031 pmol/mg protein *versus* 0.0013 ± 0.00022 pmol/mg protein). The uptake of ^{3}H-3-OMG was not medicated by OCT1 in *Xenopus laevis* oocytes or mammalian HEK-293 expression systems (data not

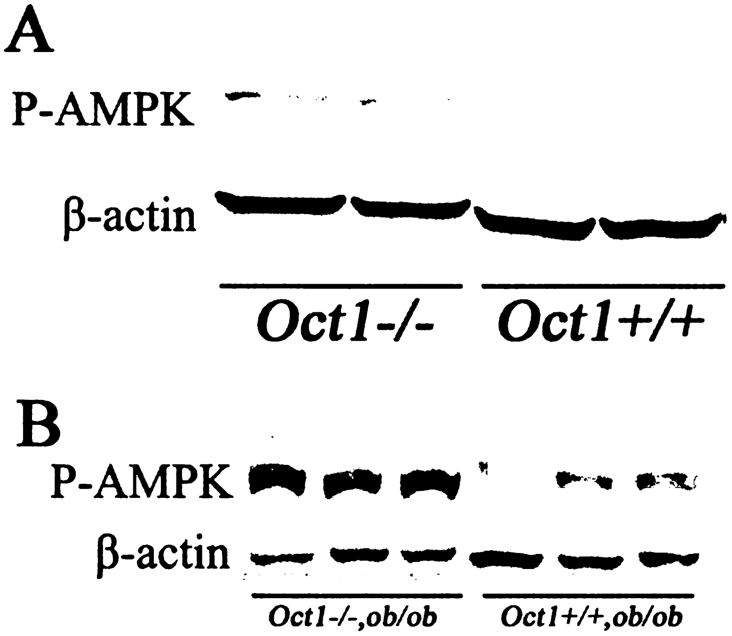


Figure 5.11. Hepatic AMPK phosphorylation in lean (A) and obese (B) mice of different *Oct1* genotypes. Mice were fasted for 16 hours before sacrifice. Liver extracts were detected with polyclonal antibodies against phospho-AMPKα (Thr172) and β-actin respectively.

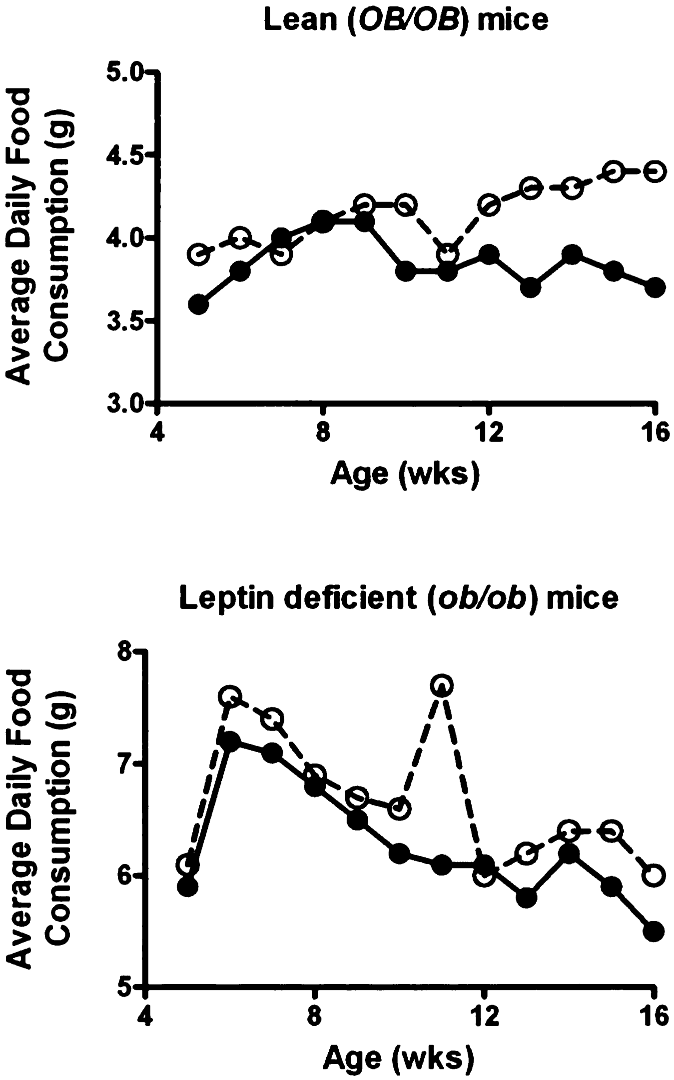


Figure 5.12. Average daily food consumption in lean (*OB/OB*, upper panel) and obese (*ob/ob*, lower panel) male mice of different *Oct1* genotypes (*Oct1-/-*, open circle; *Oct1+/+*, solid circle) during growth. Food consumption monitoring was begun one week after weaning. The weekly food consumption for a cage was calculated by subtracting the remaining amount from the amount placed at last measurement. The average daily consumption was calculated by dividing the weekly consumption by seven days and the number of mice in the corresponding cages.

shown). These data are consistent with our knowledge of the substrate specificity of Oct1, and suggest that Oct1 may not be acting on triglyceride accumulation in the liver *via* controlling the access of fatty acid and/or glucose to the hepatocyte.

Discussion

In this study, we first made a striking and unexpected observation in leptin-deficient obese mice (*ob/ob*) crossed with *Oct1* knockout mice. We observed that *Oct1* deletion markedly reduced fat accumulation in *ob/ob* mice (Figure 5.1). Along with a milder liver injury (Figure 5.3), histological studies revealed much milder fatty liver in the double mutant mice, *Oct1-/-, ob/ob*, in contrast to *Oct1+/+, ob/ob* mice, which had evidence of severe fatty liver. The plasma metabolic indices associated with lipid and glucose homeostasis (Table 5.3) were also different between *Oct1-/-, ob/ob* and *Oct1+/+, ob/ob* mice. Compared to lean *Oct1+/+* mice, lean *Oct1-/-* mice had lower body weights, liver weights, liver to body weight ratios, and hepatic triglyceride content but higher plasma glucose levels. In addition, transgenic *C. elegans* over-expressing hOCT1 had considerably more fat in the intestine (Figure 5.9). Collectively, these data suggest that Oct1 may be involved in nutrient, particularly triglyceride and glucose, homeostasis and may be important in the development of fatty liver. Our subsequent mechanistic studies indicated that OCT1 is not a transporter for fatty acid or glucose and that reduced fat accumulation in the livers of *ob/ob, Oct1-/-* mice is not explained by an increased hepatic VLDL secretion (Figure 5.10). However, it seemed that *Oct1-/-* mice had faster energy turnover than did *Oct1+/+* mice, indicated by their enhanced hepatic AMPK phosphorylation and increased food intake (Figures 5.11 and 5.12).

OCT1 has been well recognized as a transporter for certain cationic compounds including clinical drugs, environmental toxins and endogenous substances (4, 5). However, little is known regarding the physiological roles for this liver-specific, highly expressed protein. Our study, for the first time, revealed potential physiological roles for Oct1 as a participant in nutrient metabolism. As many phenotypes of gene disruption, extreme conditions are required to effectively display the phenotypes of *Oct1* deletion. In this study, we placed *Oct1-/-* mice in a leptin-deficient background that causes severe fatty liver and other metabolic syndromes. The protective effects of *Oct1* deficiency in fatty liver development were striking and unexpected. Our findings generated a fascinating question of how OCT1 regulates hepatic triglyceride content and its disruption improves fatty liver in the setting of obesity associated with metabolic syndromes.

Excess accumulation of triglycerides in fatty liver disease may result from defects in or imbalance among four metabolic pathways that determine hepatic triglyceride content: uptake of fatty acids, triglyceride synthesis, fatty acid degradation by beta-oxidation, and elimination of triglycerides through VLDL efflux (Figure 5.13) (28-30). With respect to fatty acid uptake in the liver, it appears that there are two mechanisms involved: a facilitated, transporter-mediated process for the charged fatty acid anion, and a passive diffusion process for the unionized fatty acid (31). In this study, we examined the possibility that Oct1 is a fatty acid transporter. Our results from HEK-OCT1 cells indicated that OCT1 is not such a transporter. Although speculative, hepatic fatty acid uptake may therefore be similar between *Oct1-/-* and *Oct1+/+* mice.

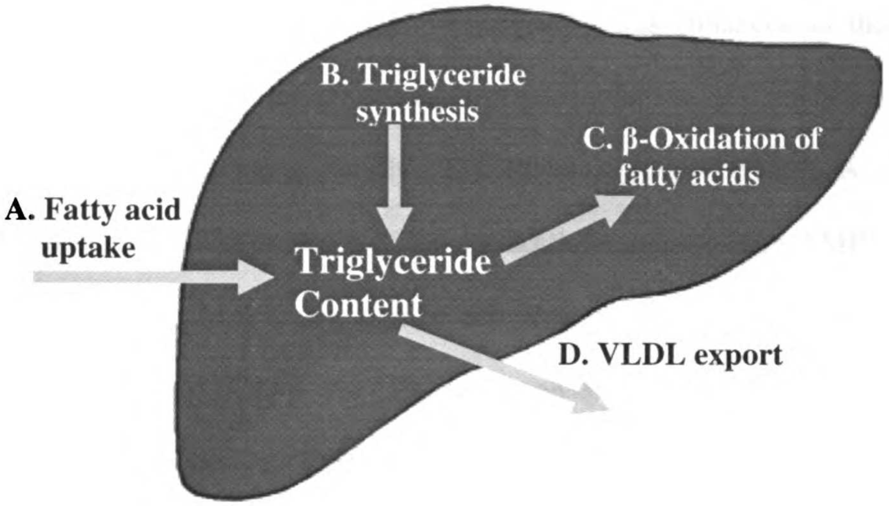


Figure 5.13. Metabolic pathways that control triglyceride content in the liver. A: Fatty acid uptake; B. Triglyceride synthesis; C. Beta-oxidation of fatty acids; D. VLDL production/export.

Increased VLDL secretion may reduce triglyceride accumulation in the liver. In the present study, *Oct1-/-, ob/ob* mice had an equal (in the male) or less (in the female) VLDL production rate, as compared to *Oct1+/+, ob/ob* mice. Therefore, the regulation of hepatic triglyceride content by Oct1 is probably not *via* the alteration of VLDL secretion.

The finding that AMPK phosphorylation was enhanced in the liver of *Oct1-/-* mouse regardless of leptin genotypes is of particular interest. AMPK is an enzyme that senses intracellular energy supply (27). Physiologically, AMPK is activated through phosphorylation in the presence of low intracellular energy (high AMP, low ATP; or high AMP/ATP ratio). AMPK can also be activated by other insults such as drug treatment (e.g. metformin) (32), hypoxia (33), and gene disruption (e.g. SCD-1 knock out mice) (34). AMPK has been recognized as a major regulator of lipid biosynthetic pathways *via* its downstream substrates such as acetyl-CoA carboxylase (ACC) (27). Furthermore, in the liver, activated AMPK stimulates fatty acid oxidation *via* the phosphorylation of carnitine palmitoyl transferase I (CPT I), and inhibits two transcription factors (SREBP-1c and ChREBP1) that enhance the expression of enzymes involved in fatty acid synthesis and gluconeogenesis (27, 28). Our data of AMPK phosphorylation suggest that the lower triglyceride accumulation in the liver of *Oct1-/-* mice may be related to decreased triglyceride synthesis and/or increased fatty acid oxidation. Consistent with this, *Oct1-/-* mice ate more food while maintaining a smaller liver to body weight ratio, as compared to *Oct1+/+* mice. However, more food consumption, on the other hand, may neutralize the effects of *Oct1* deficiency to some extent. In this study, the difference in body weight was not dramatic between *Oct1-/-* and *Oct1+/+* lean mice, and there was actually no difference in body weight between the obese mice of the two *Oct1* genotypes.

Further studies of food restriction in the models of fatty liver disease, such as *ob/ob* mice of different *Oct1* genotypes, are clearly needed. In addition to AMPK phosphorylation, the activity, protein level, and gene expression level should be compared in such studies between *Oct1-/-* and *Oct1+/+* mice for those key proteins involved in triglyceride synthesis and fatty acid oxidation and/or important hormones, such as thyroid hormone, associated with energy balance.

In this study, *Oct1* deficiency affected triglyceride homeostasis not only centrally in the liver but also peripherally in fat tissues (Figure 5.4). Because Oct1 is predominantly expressed in the liver with little in adipose tissues (10, 11), the effect of *Oct1* deficiency on peripheral adiposity may be secondary to that in the liver. Oct1 may function to cause fat redistribution in the body. In *Oct1+/+, ob/ob* mice, more fat was accumulated in the liver and less in fat tissues, and *vice versa* in *Oct1-/-, ob/ob* mice. Such paradox of fat distribution has been reported for mice over-expressing SREBP-1a, a transcription factor that transcriptionally enhances the expression of genes involved in cholesterol and triglyceride synthesis (35), and mice deficient in SREBP cleavage-activating protein (SCAP) gene in the liver (36). Hepatic overexpression of SREBP-1a resulted in massive fatty liver, while the amount of white adipose tissue such as the epididymal fat pad decreased progressively in the transgenic mice (35). The SCAP transports SREBPs from ER to Golgi where the active domains of SREBPs are released to enhance fatty acid and cholesterol synthesis. In contrast to the SREBP-1a transgenic mice, mice with conditional SCAP deficiency in the liver exhibited substantial reductions in hepatic lipid synthesis that was balanced by an equal increase in nonhepatic tissues, primarily adipose tissue (36).

Consistent with less triglyceride accumulation in the liver, the plasma activities of enzymes associated with liver injury and the plasma insulin levels were significantly lower for *Oct1-/-, ob/ob* mice in comparison to *Oct1+/+, ob/ob* mice. Moreover, male *Oct1-/-, ob/ob* mice had lower plasma cholesterol levels, and the females had lower plasma triglyceride levels. In general, when referred to the values of the metabolic indices in lean mice, these data suggested improvement of triglyceride or nutrient homeostasis by *Oct1* deficiency. However, our data of plasma glucose levels was somewhat contrary. For male mice, when compared to those of respective *Oct1+/+* control mice, both lean and obese *Oct1-/-* mice had significantly higher fasting glucose levels in the plasma. The levels were also higher for female lean *Oct1-/-* mice. Although the underlying mechanism is unclear, these data suggest that *Oct1* deficiency may result in decreased or impaired secretion of insulin. In our recent preliminary observations, male *Oct1-/-, ob/ob* mice had more severe polyuria than male *Oct1+/+, ob/ob* mice (data not shown). Therefore, it is possible that *Oct1* deficiency improves fatty liver disease while aggravating diabetes in *ob/ob* mice.

As described above, there were gender differences with respect to the effects of *Oct1* deficiency on lipid and glucose homeostasis in this study (Table 5.3). In addition, the effects may be age-dependent. When obese *Oct1-/-* and *Oct1+/+* mice were sacrificed and compared at different ages (11, 14, 16 and 20 weeks), the liver weight, liver to body weight ratio and liver-associated enzyme tests tended to be different between the two genotypes at all ages examined, but were most apparent at the age of 20 weeks (data not shown since sample sizes were small for the younger mice). The reasons for the gender and the possible age differences are unknown. Clues may be obtained from further

studies exploring the molecular mechanisms responsible for the regulation of triglyceride and glucose by *Oct1* deficiency.

At present, it is difficult to understand the molecular mechanism by which Oct1 plays a role in lipid and glucose homeostasis as the data were totally unexpected. It has been reported that Oct1 expression is downregulated in animal models of cholestasis and diabetes (37, 38). The downregulation may be secondary to the diseases. Alternatively, it may be a protective feedback mechanism of attenuating Oct1 function to improve hepatic nutrient metabolism. Because Oct1 has been characterized as a membrane transporter protein, a logical hypothesis for the phenotypes we observed is that an endogenous physiologic substrate of Oct1 regulates hepatic triglyceride and/or glucose homeostasis. Some endogenous compounds including choline, dopamine, epinephrine, norepinephrine, and histamine have been reported to be Oct1 substrates (12, 13) and probably play a role in Oct1-mediated hepatic triglyceride homeostasis. Other possibilities are endogenous analogs or derivatives of these substrates. Further studies may begin with the known compounds and their endogenous analogs/derivatives to identify a physiologic Oct1 substrate, if any, which is involved in hepatic triglyceride and glucose metabolism. It is also possible that Oct1 serves as a receptor and/or is a physical component in the metabolic pathways responsible for hepatic triglyceride and glucose metabolism.

The present study provides preliminary evidence for a role of Oct1 in triglyceride homeostasis in the liver. However, it has important limitations. Our major findings were obtained from the obese mice which were not genetically pure. The mice were crossed from C57BL/6J and FVB/N inbreeds, and had an average 50% background for each strain. A recent study showed that C57BL/6J *ob/ob* mice have more severe hepatic

steatosis but much milder hyperglycermia, as compared to FVB/N *ob/ob* mice (39). Therefore, it is possible that the observed phenotypes were due to the genetic background differences between C57BL/6J and FVB/N. That is, a genetic difference may cosegregate with the *Oct1* allele during breeding. However, we did experiments on lean *Oct1-/-* and *Oct1+/+* mice that have more than 97% background for FVB/N or C57BL/6J. Although there were milder effects, the results were consistent with those of *ob/ob* mice in this study. Future studies in the *ob/ob* mice of a pure FVB/N or C57BL/6J background are thus warranted. In addition, the observed phenotypes have not been fully characterized. For example, more mice should be included to determine the age- and gender-dependent phenotype differences. The magnitude of insulin resistance may be compared between *Oct1-/-, ob/ob* and *Oct1+/+, ob/ob* mice. The effects of *Oct1* deficiency may be studied with another fatty liver model such as mice fed a high fat diet.

In summary, we show here that *Oct1* deficiency improved fatty liver in leptin-deficient obese mice and had effects on homeostasis of nutrients such as triglyceride and glucose. In addition to its function as a drug transporter, Oct1 thus has physiological roles. Identification of molecular events in the regulation of nutrient metabolism by Oct1 could bring further insight into the mechanisms of development of metabolic syndromes such as fatty liver, insulin resistance, and diabetes. Immediate studies are required to characterize the observed phenotypes in more detail and to decipher the underlying mechanisms.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Organisms have evolved complicated transporter systems to facilitate absorbing essential nutrients, excreting metabolic waste products, and eliminating environmental toxins. Many clinically used drugs have been found to be translocated by some of these transporter systems. Transporter proteins capable of translocating a variety of structurally diverse compounds are often termed xenobiotic transporters, although they transport both endogenous and exogenous compounds. Among xenobiotic transporters, those in the SLC22A family are of great interest because they are responsible for the translocation of many drugs that are organic cations, anions or zwitterions (1, 2). The SLC22A family has at least three subfamilies: organic cation transporters (OCTs), organic anion transporters (OATs), and novel organic cation transporters (OCTNs). Each subfamily has several members. The focus of this dissertation, OCT1, encoded by *SLC22A1*, has two paralogs: OCT2 and OCT3.

Significant progress has been made in understanding the molecular, cellular, physiological and pharmacological role of the OCTs in the past decade (Chapter 1). The greatest advance is the cloning of the three members of the OCT subfamily from different species. Following this, the transport mechanisms, the tissue distribution patterns and cellular localizations, and the specificity for substrates and inhibitors have been extensively investigated. Recently, studies have focused on the regulation of activity and expression of OCTs. Knockout mouse models for each of the OCTs have provided a wealth of understanding of the physiological and pharmacological function of these

transporters. The genetic variation of human *OCT* genes has been increasingly studied. With the great research progress, it is a time to further understand their clinical importance and physiological roles of OCTs.

In Chapter 2, as a first step in understanding whether genetic variation in human *OCT1* (*SLC22A1*) contributes to interindividual differences in drug response, the genetic variants of *OCT1* were identified and functionally characterized. Thirty-seven variants were discovered in *OCT1* from 247 DNA samples from ethnically diverse populations. Functional analysis was conducted in *Xenopus laevis* oocytes for all of the 15 non-synonymous variants. Five were found to reduce (OCT1-R61C and OCT1-P341L) or eliminate (OCT1-G220V, OCT1-G401S, OCT1-G465R) the transporter function for the model substrate MPP^+, whereas one (OCT1-S14F) increased transporter function. Biochemical mechanisms for changes in transporter function were further revealed for certain variants. Four of the five decreased-function variants exhibited allele frequencies of at least 1% in at least one ethnic population. This study, together with studies by others (3-5), suggests that genetic variation in OCT1 represents a potential factor influencing drug disposition.

To choose single nucleotide polymorphisms (SNPs) for genetic association studies, algorithms and criteria to predict the function of nonsynonymous variants are being developed (6, 7). In Chapter 2, the changes in OCT1 variants were evaluated by different criteria (chemical change, evolutionary conservation, and amino acid substitution scoring matrices such as BLOSUM62 and SIFT). All of the five variants that decreased function altered evolutionarily conserved amino acid residues whereas only two of the ten other variants affected evolutionarily conserved residues. In general,

variants with decreased function had amino acid substitutions that resulted in more radical chemical changes (higher Grantham values) and were less evolutionarily favorable (lower Blosum62 values) than variants that maintained function. The results provided strong experimental evidence that changes at evolutionarily conserved positions of transporter proteins are strong predictors of decreased function. This study, along with the analysis of genetic variants in other transporters by this laboratory (8, 9), suggests that a combination of evolutionary conservation and chemical change is a strong predictor of function.

Transporters may regulate the pharmacological and toxicological effects of drugs as they may control their distribution to tissues responsible for the effects. As for drug influx transporters, however, direct evidence was limited prior to the start of this dissertation research. In Chapter 3, efforts were aimed at providing evidence that liver-specific OCT1 was a determinant of the pharmacological effects of its substrate metformin. Metformin is a very important anti-diabetic agent, being a top prescribed drug in the U.S. market (10). The drug has been reported to act through the intracellular "energy-sensor", adenosine monophosphate (AMP)-dependent kinase (AMPK) (11, 12). We found that Oct1 was a determinant of AMPK activation by and cellular response to metformin in the cell lines studied. Deletion of *Oct1* resulted in a remarkable loss of metformin-mediated AMPK activation and inhibition of gluconeogenesis in primary hepatocytes from mouse. The glucose-lowering effect of metformin was abolished in *Oct1*-deficient mice on high-fat diets. Clinical studies demonstrated that the effects of metformin on glucose levels after glucose tolerance tests were significantly lower in individuals carrying reduced function polymorphisms of OCT1. Collectively, the data

indicate that OCT1 is required, at least in part, for the therapeutic effects of metformin and by inference, that metformin has an intracellular target. Interestingly, results from Wang *et al.* suggest that distribution of metformin into the liver by Oct1 may also enhance its toxicity (13). Our findings and those from Wang *et al.* are important to further understand molecular mechanisms of metformin action. Furthermore, our studies suggest that the development of new drugs or the modification of marketed drugs, specifically targeted to the liver through OCT1, represents a novel strategy for achieving liver specificity and minimizing unwanted effects in other tissues.

Studies presented in Chapter 3 were also particularly designed to determine whether the genetic variants of OCT1 alter the response to metformin in cells and in humans. The genetic variants of OCT1 that had been functionally characterized (see Chapter 2) were re-evaluated with respect to their transport function for metformin. Compared to OCT1-reference, seven OCT1 variants exhibited significantly reduced metformin uptake in cells. The data indicate that the OCT1 variants exhibit differences in substrate specificity. For example, OCT1-S14F exhibited increased function for MPP^+ but reduced function for metformin. Consistent with the uptake function, phosphorylation of AMPK and ACC by metformin was reduced in cells expressing the reduced uptake variants of OCT1. In the clinical study, similar plasma glucose levels for baseline oral glucose tolerance test (OGTT) were observed between the volunteers who carried at least one of the reduced function variants (OCT1-R61C, G401S, 420del, and G465R) and the volunteers who only carried reference OCT1. In contrast, following metformin dosing, the volunteers carrying OCT1 variants had significantly higher plasma glucose levels for most of the sampling time points during OGTT compared to the ones not carrying a

reduced function variant. These differences resulted in a significantly greater glucose exposure in the group carrying OCT1 variants compared to the group carrying only reference OCT1. Our findings indicate that *OCT1* polymorphisms modulate cellular and clinical response to metformin. As presented in Chapter 2 and by others (3-5), the human OCT1 gene is highly polymorphic, with common polymorphisms causing function changes. On the other hand, clinically, there is enormous variation in response to metformin in diabetic patients (14, 15). Our study suggests that genetic variation in OCT1 may contribute to the wide variation in response to this critically important therapeutic agent. Although speculative, genotyping OCT1 may inform metformin therapy. For example, a higher dose of metformin may be indicated in individuals with reduced function OCT1 variants.

In Chapter 4, *in vivo* pharmacokinetic and pharmacodynamic studies of metformin in mice and healthy volunteers are presented. The pharmacokinetic parameters of oral metformin such as AUC, oral volume of distribution and C_{max} were significantly different between the individuals who carry OCT1-R61C, G401S, 420del, or G465R and those who only carry reference OCT1 alleles. A similar trend was observed in *Oct1-/-* and *Oct1+/+* mice. These data provide evidence that OCT1 function has an impact on the pharmacokinetics of metformin. With respect to metformin pharmacodynamics, the glucose-lowering effects of metformin in glucose tolerance test (GTT) were significantly reduced in *Oct1-/-* mice compared to *Oct1+/+* mice. Further, the attenuation of insulin secretion by metformin was significantly impaired in the individuals who carried the variants compared to those who only carried reference OCT1 alleles. The pharmacodynamic findings reinforced those in Chapter 3 and suggest that OCT1 function

may have a considerable impact on the pharmacodynamics of metformin. In humans, OCT1 is predominantly expressed in the liver (16, 17). Our results may be extended to other drugs that are OCT1 substrates and for which the liver is important in disposition and/or response. In particular, OCT1 function or genotype may have a considerable effect on the systemic pharmacokinetics of substrates that are extensively metabolized in the liver. Such an effect may be translated to an effect on drug action that occurs not only in the liver but also in other target tissues. Our findings will lead to an increased awareness of potential OCT1-mediated drug actions and drug-drug interactions, and may therefore facilitate a more rational use of drugs.

Although OCT1 has been well recognized as a drug transporter, its physiological roles are still unclear. *Oct1* knockout mice appear to be normal and have no obvious phenotypic abnormalities (18). It has been proposed that the phenotypes related to *Oct1* deficiency may be revealed under specific extreme conditions (18). In Chapter 5, we first observed that *Oct1* deletion markedly reduced fat accumulation in the liver of *ob/ob* mice. This is an unexpected novel finding. We then performed a series of assays and measurements in obese (*ob/ob*) and lean (*OB/OB*) mice in the presence or absence of *Oct1* alleles. The data suggest that Oct1 may be involved in nutrient, particularly triglyceride and glucose, homeostasis and may be important in the development of fatty liver. Our subsequent mechanistic studies indicated that OCT1 is not a transporter for fatty acids or glucose and that reduced fat accumulation in the livers of *ob/ob*, *Oct1-/-* mice is not explained by an increase in hepatic VLDL secretion. However, it seems that *Oct1-/-* mice have a greater energy turnover than do *Oct1+/+* mice, indicated by their lower body weight, enhanced hepatic AMPK phosphorylation and increased food intake in the male *Oct1-/-* mice we studied. The studies presented in Chapter 5 represent a preliminary characterization. Immediate studies are required to characterize the observed phenotypes in more detail and to decipher the underlying mechanisms. However, our findings provide first clues to the physiological roles of OCT1. Identification of molecular events in the regulation of nutrient metabolism by OCT1 might bring further insight into the mechanisms of development of metabolic syndromes such as fatty liver, insulin resistance, and diabetes. Therefore, in addition to serving as a drug transporter, OCT1 might be a drug target *per se*.

Research in this dissertation has opened up new areas of inquiry. With respect to pharmacokinetics and pharmacodynamics, in addition to metformin, what other drugs are affected by OCT1 genotype? Is OCT1 also associated with risk to any disease? It may be worthwhile to investigate the association of genetic polymorphisms of OCT1 with fatty liver, diabetes, or other metabolic syndromes because of the potential involvement of OCT1 in hepatic triglyceride homeostasis (Chapter 5). For example, OCT2 polymorphisms have been reported to be associated with essential hypertension (19). We demonstrated that OCT1 is a determinant of the desired pharmacological effects of metformin in animals and in healthy volunteers. The findings need to be reproduced in diabetic patients by conducting retrospective and prospective clinical studies. Such clinical studies should also evaluate whether OCT1 genotype is a predictor of meformin induced glucose control in patients. The *Oct1-/-* mouse model should be helpful to identify an intracellular target(s) for metformin. Giving the huge, specific expression of OCT1 in the liver, candidate compounds with pharmacological activities may be synthesized to target OCT1 to achieve liver specific drug delivery. The preliminary

findings of the involvement of OCT1 in nutrient homeostasis need to be further confirmed. Studying OCT1 physiology and its related pathology will reveal the roles of OCT1 in the metabolic pathways for triglycerides, glucose and cholesterol. Investigations of the role of OCT1 in the pathogenesis of fatty liver, insulin resistance, diabetes and other metabolic syndromes will be of great interest to pursue. Finally, our studies suggest that similar studies examining the physiological and pathological roles of other so-called xenobiotic transporters are warranted.

The studies presented in this dissertation, from basic mechanistic studies to clinical studies, together provide a paradigm of pharmacogenetic research for xenobiotic transporters.

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