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Hepatic OATP Transporter and Thyroid Hormone Receptor Interplay Determines Cholesterol and Glucose Homeostasis

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Abstract

The role of Organic Anion Transporting Polypeptides (OATPs), particularly the members of OATP1B-subfamily, in hepatocellular handling of endogenous and exogenous compounds is an important and emerging area of research. Using a mouse model lacking *Slco1b2*, the murine ortholog of the OATP1B-subfamily, we previously demonstrated that genetic ablation causes reduced hepatic clearance capacity for substrates. In this report we focused on the physiological function of the hepatic OATP1B transporters.

First we studied the influence of the *Oatp1b2* deletion on bile acid metabolism showing that lack of the transporter results in a significantly reduced expression of *Cyp7a1* the key enzyme of bile acid synthesis, resulting in elevated cholesterol levels after high dietary fat challenge. Furthermore, *Slco1b2*^{-/-} mice exhibited delayed clearance after oral glucose challenge resulting from reduced hepatic glucose uptake. In addition to increased hepatic glycogen content, *Slco1b2*^{-/-} exhibited reduced glucose output after pyruvate challenge. This is in accordance with reduced hepatic expression of PEPCK in knockout mice. We show this phenotype is due to the loss of liver-specific *Oatp1b2*-mediated hepatocellular thyroid hormone entry, which then leads to reduced transcriptional activation of target genes of hepatic thyroid hormone receptor (TR) including the prior mentioned *Cyp7a1* and *Pepck*, but also *Dio1* and *Glut2*. Importantly, we assessed human relevance using a cohort of archived human livers where OATP1B1 expression was noted to be highly associated with TR target genes, especially for GLUT2. Furthermore, GLUT2 expression was significantly decreased in livers harboring a common genetic polymorphism in *SLCO1B1*.

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Conclusion—Our findings reveal that OATP1B-mediated hepatic thyroid hormone entry is a key determinant of cholesterol and glucose homeostasis.

Keywords

Liver; hepatocytes; drug transporter; OATP1B1; thyroid hormones

Transporter expressed in the plasma membrane of eukaryotic cells function as gatekeepers for cellular homeostasis. Among solute uptake transporters, members of the Organic Anion Transporting Polypeptide (OATP) superfamily have been widely studied for their role in drug disposition. In particular OATP1B1 (*SLCO1B1*) and OATP1B3 (*SLCO1B3*), members of the OATP1B subfamily have been shown to facilitate hepatic uptake of a variety of exogenous and endogenous compounds (1;2). Bile acids, thyroid hormones, and estrogen metabolites are widely accepted as endogenous substrates of both transporters. In addition, there is an expanding list of xenobiotic substrates including several drugs in clinical use. For OATP1B1 various single nucleotide polymorphism (SNPs) have been described, that are linked to diminished transport activity *in vitro* (3). Importantly, presence of those genetic variants translates into altered drug disposition *in vivo* (4). The clinical relevance of OATP1B1 to drug response has been highlighted by its emerging role as a biomarker for statins-induced muscle injury. We now know *SLCO1B1* polymorphisms result in increased plasma levels of statins that might result in decreased pharmacological effects, while profoundly increasing the risk for muscle toxicity (5;6).

However, little is known about the physiologic role of OATP1B transporters. Recently we showed that targeted disruption of the murine ortholog of the human OATP1B transporters namely the *Slco1b2* gene resulted in a significant reduction of hepatic uptake of known substrate drugs, consistent with the expected role of Oatp1b2 in drug disposition (7). We now report an unexpected physiological function of this transporter through the linkage of Oatp1b2 to liver-specific delivery of thyroid hormones, thereby affecting gene expression of hepatic thyroid hormone receptor targets linked to cholesterol and glucose homeostasis.

Experimental Procedures

Animals

All of the described experiments were performed using male mice aged between 8 and 12 weeks.

Real-time PCR

For quantitative real-time PCR mRNA was isolated using the RNeasy®Mini Kit (Qiagen, Valencia, CA). After cDNA synthesis expression determined using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) (detail in Supplemental Material).

Immunohistochemistry

For immunohistochemistry paraffin embedded tissue slides were stained using a primary anti-Glut2 antibody (1:150) (Abcam, Cambridge, MA) and fluorescence or HRP-labeled secondary antibodies (Vectorstain ABC-Kit, Vector Laboratories, Burlingame, CA). Staining was detected using a Nikon light, or fluorescence microscope, respectively (detail in Supplemental Material).

Western Blot analysis

Proteins were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes (Invitrogen, Carlsbad, CA), and protein expression determined by the use of indicated primary antibody (Supplemental Table 1). Binding of the antibody was detected using HRP-labeled secondary antibodies (BioRad, Hercules, CA) and the AmershamTM ECL Plus Western Blotting Detection Reagents (GE Healthcare, Baie d'Urfe, Quebec, Canada). Chemiluminescence was determined using a KODAK ImageStation 4000MM (Mandel, Guelph, ON, Canada).

Dietary high fat Challenge

Animals were fed *ad libitum* with Western Diet (TestDiet, Richmond, IN) containing 16.8% protein, 6.5% fiber, 48% carbohydrates and 20% fat. After 6 weeks of feeding wildtype and *Sleo1b2*^{-/-} mice were sacrificed and blood samples were collected.

Blood Biochemistry

The measurement of cholesterol and TSH was performed at Charles River Laboratories (Wilmington, MA). Total and free thyroxine and triiodothyronine in plasma were determined using ELISA kits from Alpha-Diagnostics (San Antonio, TX). Insulin levels were determined using the UltraSensitive Mouse Insulin ELISA kit[®] (Crystal Chem Inc., Downers Grove, IL). Total bile acids or 7- α -hydroxy-4-cholesten-3-one were determined using a commercially available colorimetric assay (BioQuant, San Diego, CA) or mass-spectrometry (detail in Supplemental Material). Glucose tolerance testing and pyruvate challenge were carried out using 2g/kg of glucose or pyruvate. Glucose levels were determined using a glucometer (OneTouch[®], LifeScan Inc., Milpitas, CA).

Determination of TH Levels in Liver

For TH extraction tissue was homogenized in methanol. After addition of chloroform (2:1) and centrifugation (15min, 1900xg, 4°C), pellets were re-extracted with a chloroform/methanol (2:1) –mixture. Both supernatants were combined and further extracted with chloroform: methanol: water (8:4:3) and 0.05% CaCl₂. The mixed solution was centrifuged (10min, 800xg, 4°C). Lower apolar phase was re-extracted with chloroform: methanol: water (3:49:48). The upper polar layers were pooled and thyroxine was detected by EIA (Alpha-Diagnostics).

Parenteral [³H]-Glucose Distribution

Hepatic glucose uptake was assessed in mice treated i.v. with 0.5 mg/kg bodyweight glucose supplemented with 1 μ Ci of [³H]-D-glucose. After three minutes, blood samples were collected, and liver tissue was harvested, followed by homogenization in PBS. 200 μ l of the homogenate were bleached using an equal volume of a 3%-NaClO-solution, afterwards 1ml of water was added. Plasma or tissue homogenate radioactivity was determined using a Liquid Scintillation counter (Liquid Scintillation Analyzer, Tri-Carb 2900TR, PerkinElmer, Waltham, MA).

Determination of hepatic glycogen content

Periodic Acid Schiff's staining was performed using a commercially available staining kit (Sigma-Aldrich). Hepatic glycogen content was measured calorimetrically as described previously (8). After sample and standard preparation, absorption at 490nm was determined using a spectrometric plate reader (MultiskanSpectrum, Thermo-Fisher, Waltham, MA).

Heterologous expression experiments

Heterologous expression experiments were performed to measure accumulation of the endogenous substrate estrone-3-sulfate (E₁S). HeLa cells were infected with vtf-7 virus. After 30min incubation at 37°C 1 µg of the plasmids was transfected into the cells using Lipofectin® (Invitrogen). After subsequent culture overnight transport experiments were performed. ³[H]-E₁S accumulation was determined after 10min incubation using a scintillation counter.

Luciferase Assay

OATP-mediated cellular entry of TH was assessed monitoring the TRβ associated transactivation of a DIO1 promoter containing luciferase reporter construct. Luciferase activity was determined after treatment with medium supplemented with 5% charcoal-stripped FBS and 100nM thyroxine or 100nM triiodothyronine using the commercially available Luciferase Assay System® (Promega) (detail in Supplemental Material).

Primary Human Hepatocytes

Freshly isolated human hepatocytes from 3 different individuals were obtained from Lonza Verviers SPRL (Verviers, Belgium). Upon arrival, the medium was changed and treatment with 10nM triiodothyronine or 10nM thyroxine started. After 24hrs treatment hepatocytes were harvested for mRNA isolation. The samples were used for quantification of DIO1 and GLUT2, and 18S expression by real-time PCR.

Gene expression analysis from Human Liver Samples

Human liver mRNA expression data were obtained from GEO GSE9588 (9). These data are based on expression profiling using a custom Agilent 44,000 feature microarray composed of 39,280 oligonucleotide probes targeting transcripts representing 34,266 known and predicted genes, including high-confidence, noncoding RNA sequences in a human liver cohort comprising 427 subjects. 423 samples were included into the following linear regression analysis outlier samples were removed based on global expression visualized by PCA as described previously (9)

SLCO1B1 and SLCO1B3 genotyping

Human liver tissue (n=60) provided by the Liver Tissue Procurement and Distribution System (NIH-Contract #N01-DK-9-2310) and by the Cooperative Human Tissue Network was processed through St. Jude Liver Resource. Extracted DNA was genotyped for c.388A>G (rs2306283) and c.521C>T (rs2306283) in SLCO1B1 and c.334G>T (rs4149117) and c.699G>A, (rs7311758) in *SLCO1B3*. Genotyping was performed by direct sequencing (Supplemental Tab. 1).

Statistical Analysis

Unpaired t-test was used, but for determination of the impact of *Slco1b2* deletion on the time course of the oral glucose tolerance test, the cell based Luciferase assay and the transport experiment which were statistically validated using ANOVA. Associations between gene expression in human livers was evaluated using linear regression modeling determining the linear regression coefficient r^2 , and performing an F-test. The degree of linear relationship of two variables is reflected by the Pearson product-moment correlation coefficient (*Pearson r*). The impact of genotypes was evaluated using Kruskal-Wallis one-way ANOVA. Finally, p-values were adjusted according to Benjamini-Hochberg False Discovery Rate, adjusted p-values <0.05 were defined statistically significant (10). Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Dysregulation in cholesterol and bile acid homeostasis in *Slco1b2*^{-/-} mice

Since bile acids (BA) are known substrates of the OATP1B transporters (11), we first examined whether loss of *Oatp1b2* has an effect on BA homeostasis. Associated with the deletion of *Slco1b2* was a modest, but not statistically significant reduction of total BAs in livers of 10 weeks old mice, while plasma levels were 3-fold lower compared to wildtype mice (Figure 1A). Recent data by Csanaky et al. using a similar mouse model showed significantly increased levels of serum total bile acids comparing wildtype and *Slco1b2*^{-/-} mice aged between 36 and 48 weeks (12). When we examined hepatic expression of Cyp7a1, the key enzyme involved in bile acid formation from cholesterol, consistent with our first hypothesis, *Slco1b2*^{-/-} mice exhibited significantly lower mRNA expression of Cyp7a1 (Cyp7a1 relative to wildtype, wildtype 1.04 ± 0.28 (n=10), *Slco1b2*^{-/-} 0.45 ± 0.29 (n=10), adj. p-value=0.009). This finding is similar to that shown by Csanaky et al. (12). Similar results were obtained when the protein level of Cyp7a1 was determined. (Figure 1B). Consistent with reduced Cyp7a1 expression, there was a 1.7-fold higher increase in plasma cholesterol levels in knockout mice after exposure to high fat diet (Figure 1C). However, when we determined the level of the bile acid precursor 7- α -hydroxy-4-cholesten-3-one, which is thought to be a surrogate marker of CYP7A1 activity in humans (13), we did not observe statistically significant differences between wildtype and knockout animals (Supplemental Figure 1).

Altered hepatic glucose handling in *Slco1b2*^{-/-} mice

Since Cyp7a1 expression is regulated by THs (14) and members of the OATP1B subfamily are known to be capable of transporting thyroxine (T₄) and triiodothyronine (T₃) (15), we assumed that the observed dysregulation was the result of a functionally important role of *Oatp1b2* to hepatic TH response, so that other TH regulated pathways would be affected. We therefore assessed glucose homeostasis. Oral glucose tolerance tests revealed that *Slco1b2*^{-/-} mice exhibited a significantly reduced ability to lower glucose levels associated with a significant delay in glucose removal (Figure 2A). Moreover, knockout animals showed a trend for increased glucose levels (glucose $\pm$ SEM, wildtype 117.41 ± 1.35 , n=15; *Slco1b2*^{-/-} 131.60 ± 1.46 ; n=15; adj. p-value=0.056), that was not related to changes in insulin levels after 3hrs of fasting (insulin $\pm$ SEM, wildtype 0.72 ± 0.08 , n=5, *Slco1b2*^{-/-} 0.62 ± 0.09 ; n=5; adj. p-value=0.441). Subsequently, the hepatic uptake of [³H]-D-glucose was determined 3min post intravenous administration revealing significantly reduced glucose accumulation in livers of *Slco1b2*^{-/-} compared to wildtype animals, yet no differences were observed in plasma levels (Figure 2B). To test whether gluconeogenesis is affected we determined glucose levels after pyruvate challenge. While wildtype mice responded with significantly increased blood glucose levels 15min after intraperitoneal pyruvate injection, knockout animals did not (Figure 2C). Hepatic glucose catabolism appeared similarly disturbed, as shown by Periodic Acid Schiff's staining of livers revealing significant higher glycogen accumulation in hepatocytes of knockout animals, which was confirmed by calorimetric analysis (Figure 2D, 2E).

Thyroid hormone status in *Slco1b2*^{-/-} mice

Examination of the hepatic expression of known TR target genes further supported the reduced hepatic TH activity, showing significant down-regulation of 5'-Deiodinase type 1 (Dio1), and Phosphoenolpyruvate carboxykinase (Pepck) (Figure 3A, B). In addition, determining the TH status comparing wildtype and knockout mice revealed significantly reduced levels of fT₄ in livers associated with significantly elevated plasma levels translating into lower liver-to-plasma ratios of the latter (Figure 4A). However, no significant alterations in fT₃ levels were detected (Figure 4A). Similarly no difference was

seen for the pituitary thyroid stimulating hormone (TSH [$\mu\text{g/ml}$]) comparing wildtype (0.12 ± 0.01) and *Slco1b2*^{-/-} mice (0.17 ± 0.09).

Oatp1b2 transport activity and thyroid hormone effects in vitro

The interaction of mouse Oatp1b2 with TH was determined performing *in vitro* experiments using Oatp1b2-overexpressing cells. In accordance with our assumption THs significantly inhibited the Oatp1b2 mediated uptake of the known substrate estrone-3-sulfate (E₁S). E₁S-uptake (180.16 ± 14.64 % of vector control) was significantly reduced by concomitant exposure with 100nM T₄ (151.99 ± 5.60 %), 100nM T₃ (112.46 ± 15.52 %) or 100nM rT₃ (96.64 ± 16.30 %) (adj. p-value=0.0007). To test whether THs are indeed substrates for mouse Oatp1b2, we used a cell-based reporter gene assay, whose luciferase signal was driven by the TR-sensitive DIO1-promoter. Experiments showed that co-expression of Oatp1b2 substantially increased activation of the DIO1-reporter when cells were treated with T₄ or T₃ (Supplemental Figure 2A & 2B). It should be noted that expressed level of LXR target genes including Abcg5, Abcg8, Abca1, and Srebf1 did not differ between wildtype and *Slco1b2*^{-/-} mice (Supplemental Figure 3)

Reduction of Hepatic Glut2 (Slc2a2) in Slco1b2^{-/-} mice

The glucose transporter Glut2 (*Slc2a2*) is a known TR target gene (16) and facilitates hepatocellular glucose uptake thereby regulating expression of enzymes involved in glucose homeostasis in liver (17). Assessing isolated human hepatocytes for TH mediated regulation of GLUT2 showed significant induction by T₃ and T₄, respectively (Figure 5C). Detection of Glut2 in mouse liver revealed significantly lower expression in knockout compared to wildtype mice (Figure 5A, 5B, 5D). Importantly, pancreatic expression of Glut2 did not differ between wildtype and *Slco1b2*^{-/-} animals (Supplemental Figure 4), indicating that changes in Glut2 were liver-specific, consistent with the liver-specific function of Oatp1b2.

Expression of OATP1B transporters and TR- target genes in human liver samples

We tested whether OATP1B1 transporter expression was related to GLUT2 levels in human liver tissue. We found that expression of GLUT2 tended to follow OATP1B1 protein levels (Figure 6A, Supplemental Figure 5). Next, we assessed the mRNA expression of OATP1B subfamily transporters (OATP1B1 and OATP1B3) in a larger cohort of 423 human liver samples and noted a remarkable correlation of OATP1B1 and GLUT2 expression (Figure 6B) and a much lower association between OATP1B3 and GLUT2 expression ($r^2=0.3521$, *Pearson* $r=0.5934$, adj. p-value=0.001) (Supplemental Figure 6). Similar correlations were observed between expression of OATP1B1 and other TR target genes including CYP7A1 ($r^2=0.3352$; *Pearson* $r=0.5789$, adj. p-value=0.002), PEPCK ($r^2=0.4833$, *Pearson* $r=0.6952$, adj. p-value=0.001), and DIO1 ($r^2=0.3255$, *Pearson* $r=0.5705$, adj. p-value=0.001), while the correlation to TR-target genes and OATP1B3 was much lower (Supplemental Figure 7).

SNPs in human SLCO1B1 affect GLUT2, DIO1 and PEPCK expression

SNPs associated with impaired transport activity of OATP1B1 have been previously described (3). In addition, SNPs in OATP1B3 are known to exist, although not consistently associated with functional difference (18). Since mouse Oatp1b2 has sufficient sequence similarity to both human OATP1B1 and 1B3, we genotyped livers (n=60) for *SLCO1B1* and *SLCO1B3* polymorphisms. Subsequently, expression of TH target genes was examined in relation to the transporter genotypes. As shown in Table 1 the SNPs namely *SLCO1B1* c.388A>G and c.521C>T resulting in the haplotypes *1b (c.388A>G), *5 (c.521C>T) or *15 (c.388A>G & c.521C>T) of OATP1B1 were associated with statistically significant changes in GLUT2 (adj. p-value=0.009), DIO1 (adj. p-value=0.006), and PEPCK (adj. p-value=0.010) expression in human livers. Especially the *SLCO1B1**15 haplotype was

associated with lower expression of GLUT2 (adj. p-value=0.008), DIO1 (adj. p-value=0.008) and PEPCK (adj. p-value=0.013). However, SNPs in *SLCO1B3* were not predictive of changes in expression of those target genes (Supplemental Table 2).

DISCUSSION

In this report we present data on a critical role of OATP1B transporters to liver physiology. Although we had recently shown the importance of OATP1B transporters to hepatic drug disposition using the *Slco1b2*^{-/-} mice (7), the role of this transporter to liver-specific glucose and cholesterol metabolism via modulation of TR signaling pathways, particularly with its remarkable effect on hepatic GLUT2 expression was completely unexpected. Indeed, we would have predicted that since several OATP transporters have been shown capable of mediating cellular uptake of THs (1), absence of a single isoform would not affect hepatic physiology in such a way. However, the role of transport in TH activity is supported by findings in the CNS, where mutations in MCT-8 (*SLC16A2*) have been shown to result in mental retardation due to reduced neuronal TH entry (19;20). It is remarkable that despite the multiplicity of transporters expressed in liver capable of TH transport, OATP1B transporters, both in mice and humans, appear to have a dominant role in controlling hepatic hormone status. It should be noted, that a recent study by van der Deure *et al.* suggested that OATP1B1 can also transport TH metabolites such as iodothyronine sulphates (T₄S) and that T₄S plasma levels are different in individuals harboring the *SLCO1B1* c.521C>T polymorphism, but the SNP was not associated with statistically significant changes to parent TH levels. However, their data show that the level of fT₄ at least in healthy volunteers appeared slightly higher in individuals harboring the polymorphism (521CC vs. 521CT/TT; 14.8±0.2 vs. 15.6±0.3; p=0.06) (21). In accordance with those findings we show that absence of Oatp1b2 manifests as altered hepatocellular response to THs, while plasma levels of fT₃ and TSH are unchanged and the levels of fT₄ are slightly but significantly higher in knockout mice.

Biological activity of THs is partly controlled by conversion of circulating T₄ to the more active T₃ catalyzed by intracellular iodothyronine 5'-deiodinases. In non-hepatic tissues 5'-deiodinase type II (DIO2), catalyzes the conversion of T₄ to T₃ and therefore controls the cellular activity, whereas 5'-deiodinase type I (DIO1) catalyzes the conversion of T₄ to equimolar amounts of T₃ and the biologically inactive reverse T₃ (rT₃) and thereby modulates the plasma levels of T₃ (22–24). Linking Oatp1b2 to hepatic TH function was clearly supported by our observation that expression of the widely studied and well defined TR target gene, *Dio1*, a sensitive marker of hepatic TH status (25;26), was markedly reduced in livers of *Slco1b2*^{-/-} mice.

Biological activity of THs arises from activation of intracellular nuclear hormone receptors (27). TRβ, is the predominant TR in liver, and thought to mediate the cholesterol lowering effects of a TH therapy (28;29). In fact, liver plays a central role in cholesterol homeostasis and studies of TH mimetic compounds targeting the liver have been shown to lower LDL-cholesterol and increase bile acid synthesis (30;31), and can further reduce LDL-cholesterol in dyslipidemic patients already on statin therapy (32). Our current findings would suggest Oatp1b2 is an important regulator of hepatic TH activity and its absence results in the dysregulation of cholesterol homeostasis as a result of reduced TR-mediated expression of Cyp7a1. Down regulation of Cyp7a1 in *Slco1b2*^{-/-} mice has also been recently reported by Csanaky *et al.* (12) although in their study *Slco1b2*^{-/-} mice exhibited higher serum bile acid levels. It is possible the marked age difference between the mice in that study relative to those reported here could be one explanation for the observed differences in bile acid levels. Indeed, developmental effects on bile acid pool size in rodents has been reported before (33).

Similarly, the involvement of THs in regulation of glucose homeostasis has been widely appreciated for many decades. The understanding of TH effects has been supported by *in vitro* analysis (34), and characterization of knockout mouse models linking THs to induction of hepatic gluconeogenic enzymes such as pyruvate carboxylase (PEPCK) and glucose 6-phosphatase, along with reduced insulin half-life and sensitivity (35–37). Our findings in Oatp1b2-transporter deficient mice support the linkage of hepatic TH status to glucose homeostasis resulting from reduced hepatic glucose uptake and gluconeogenesis. Dysregulation of Glut2 seems to be a major factor in TR regulation of glucose homeostasis. Indeed, it is becoming evident that glucose itself can function as a regulator of glycolysis (17). This mechanism of action appears to depend on the equilibration of glucose across the plasma membrane via glucose transporters (38). *Glut2*^{-/-} mice exhibit a diabetes phenotype characterized by hyperglycemia, relative hypoinsulinemia and high circulating free fatty acids (39). This phenotype results from impaired glucose stimulated insulin secretion in pancreatic β -islet cells (40). In *Glut2*-null mice, marked increase in hepatic glycogen content was also noted. This appears to result from elevated cytosolic glucose concentrations due to the loss of Glut2-mediated cellular efflux (41). In humans, loss of function mutations in GLUT2 have been linked to Fanconi-Bickel syndrome, a rare autosomal recessive disorder in which one hallmark feature is hepatomegaly secondary to liver glycogen accumulation (42). Therefore the mechanism by which L-thyroxine treatment results in significantly reduced hepatic glycogen content (43) is likely in part mediated by induction of Glut2 expression. Interestingly, liver specific reconstitution of Glut2 revealed that this transporter is responsible for the observed metabolic abnormalities noted in *Glut2*^{-/-} hepatocytes (41). Consistent with our data suggesting Oatp1b2-dependent TR-mediated activation of Glut2, expression of GLUT2 in human liver has been noted to be modulated by THs (16). This is supported by our findings in human hepatocytes. Reduced expression of Glut2 in mouse liver due to reduced hepatic entry of THs and activation of hepatic TR is likely to be the cause of aberrant glucose homeostasis. Importantly, expression of Glut2 in pancreatic islet cells of wildtype and *Slco1b2*^{-/-} mice did not reveal any differences since Oatp1b2 is a liver specific transporter, further strengthening our hypothesis that Oatp1b2 is linked to hepatic Glut2 expression.

An important question we addressed was whether the observed murine phenotype predicts the human situation. Oatp1b2 is an ortholog of the human OATP1B subfamily. OATP1B1 has been extensively studied and its polymorphisms are associated with impaired drug transport activity (3;4). To more fully delineate the clinical relevance of our findings, OATP1B1 and OATP1B3 expression was correlated to that of known TH target genes in a bank of human liver tissue samples. The highest correlation among 34,266 profiled genes was between OATP1B1 and GLUT2. Similar results were obtained for GLUT2 and OATP1B1 protein expression. We then hypothesized that if OATP1B1 is critical to GLUT2 expression, then known functional SNPs in this transporter would alter GLUT2 expression. Previous studies have shown that the *SLCO1B1* c.521C>T polymorphism can result in marked differences in plasma levels of substrate drugs (2) and predict statin-induced myotoxicity (6). Therefore in a subset of OATP1B1-genotype defined liver samples we determined GLUT2 expression. Consistent with our hypothesis the expressed level of GLUT2 was nearly 3-fold lower in livers of individuals harboring *SLCO1B1* c.521T>C SNP (haplotypes *5 and *15) (Table 1). Ironically, it appears that patients carrying this SNP would obtain less benefit from statin therapy due to reduced hepatic entry (5), while at the same time, at a greater risk for exhibiting aberrant glucose and cholesterol levels due to reduced hepatic TH entry, thus most likely to be prescribed statins. It will be important to determine the role of OATP1B1 to the hepatic entry TH mimetic agents such as eprotirome (32) targeting the liver, and result in reduced efficacy for carriers of OATP1B1 polymorphisms.

In conclusion, we report a physiological role of hepatic OATP1B transporters in regulating cholesterol and glucose metabolism revealed through the systematic examination of a newly created *Slco1b2*^{-/-} mouse model. Oatp1b2 in rodents and OATP1B1 in humans appear to be tightly linked to hepatic TR signaling pathways that govern glucose and cholesterol homeostasis, a proposed network is depicted in Figure 6C. Accordingly decreased activity of OATP1B1, whether due to intrinsic genetic variation or inhibition of the transporter by concomitantly ingestion of an OATP1B1 inhibitor drug (1;2), alters TH response and signaling pathways in liver, and is a heretofore unrecognized determinant of chronic diseases such hyperlipidemia and diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

OATP	organic anion transporting polypeptide
SLCO	solute carrier organic anion transporter family
T₄	thyroxine
T₃	triiodothyronine
DIO1	5' deiodinase Type 1
GLUT2	Glucose facilitating transporter 2
TR	Thyroid hormone Receptor
BA	Bile acids
TH	Thyroid hormones

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Figure 1. Dysregulation of bile acid and cholesterol homeostasis *Slco1b2*^{-/-} animals
 Total bile acid levels were determined in plasma (*left*) and liver tissue (*right*) comparing wildtype and knockout mice (**A**). Down regulation of cholesterol-7 α -hydroxylase (Cyp7a1) protein expression in liver of *Slco1b2*^{-/-} mice (**B**) and the attendant elevation in serum cholesterol levels in the knockout mice post 6-week high fat diet (**C**). Data are expressed as mean $\pm$ SD, n=5 in each group compared.

Figure 2. Dysregulation of glucose homeostasis in *Slco1b2*^{-/-} animals

Delayed plasma glucose normalization post oral glucose challenge in *Slco1b2*^{-/-} mice (**A**). Hepatic and plasmatic [³H]-glucose levels 3 min post i.v.-administration in wildtype and knockout mice (n=10) (**B**). Blood glucose levels determined prior to (white dots) and 15 min after (black dots) intraperitoneal pyruvate injection (n=8) (**C**). Hepatic glycogen content determined by Periodic Acid Schiff's staining (**D**) and by colorimetric assay (n=5) (**E**). Data are expressed as mean ± SD, * Unpaired t-test, adj. p-value<0.05, ** repeated measures ANOVA adj. p-value<0.05.

Figure 3. Expression of TR β Target genes in liver

Expression of 5'-Deiodinase type I (Dio1) (A) and Phosphoenolpyruvate carboxykinase (Pepck) (B) was assessed performing real-time PCR (Unpaired t-test, * adj. p-value <0.05, n=10).

Figure 4. Thyroid hormones and Oatp1b2 in mouse

Plasma (left column), liver (middle column), and the resulting liver to plasma ratios (right column) of free thyroxine (fT₄) and tri-iodothyronine (fT₃) were determined among knockout (black) and wildtype (white) animals (Unpaired t-test * adj. p-value < 0.05, n=9). Data are expressed as mean ± SD.

Figure 5. Hepatic Glut2 (Slc2a2) expression and response to thyroid hormones

Glucose facilitating transporter (Glut2) expression was significantly lower in *Slco1b2*^{-/-} liver samples when assessed using real-time PCR (**A**) (* adj. p-value<0.05, n=10) and Western Blot analysis (**B**). Immunohistochemical and immunofluorescent staining of liver slides comparing wildtype and *Slco1b2*^{-/-} animals (**C**). Induction of GLUT2 (**D**) and DIO1 (**E**) mRNA expression in human hepatocytes (HH) of 3 individuals treated 24hrs with thyroxine (T₄, 10nM) or triiodothyronine (T₃, 10nM) *in vitro*. Data are expressed as mean ± SD.

Figure 6. Association between OATP1B1 and GLUT2 Expression in human liver samples and role in the TR hepatic network

Correlation of OATP1B1 and GLUT2 protein expression in human liver samples (A).

Protein expression was determined by Western Blot analysis after quantification transporter expression was related to that of β -Actin. The association between OATP1B1 and GLUT2 was verified in a human liver cohort, comprised of 423 liver mRNA samples profiled using a custom Agilent 44,000 feature microarray (9) (B). Linear regression (red); 95%-prediction interval (black). A proposed pathway is depicted for Oatp1b2-mediated liver-specific TR signaling which results in altered Cyp7a1 and Glut2 expression (C). GK Glucokinase, LDL-R LDL Receptor, G6P Glucose 6-phosphate

SLCO1B1 genotype and association to thyroid hormone receptor target gene expression in human liver tissue

Human liver samples of defined SLCO1B1 genotype were further studied for TR target gene expression differences. SLCO1B1 wildtype (388A&521T) was compared to those harbouring SLCO1B1 388A>G, SLCO1B1 521T>C or SLCO1B1 388A>G and 521T>C haplotype in terms of GLUT2, CYP7A1, DIO1 and PEPCK mRNA levels. (Data are expressed as mean ± SD, Kruskal-Wallis ANOVA, adj. * p-value<0.05).

	SLCO1B1 genotype				
	388A, 521T	388G	521C	388G, 521C	
Frequency	32.5% (n=13)	25% (n=14)	7.5% (n=3)	25% (n=10)	
SLCO1B1	-0.28± 0.21	-0.25 ± 0.26	-0.26 ± 0.21	-0.34 ± 0.38	p>0.05
GLUT2	-0.18 ± 0.25	-0.25 ± 0.31	-0.45 ± 0.10	-0.70 ± 0.32 (*)	p=0.004
CYP7A1	-1.02± 0.59	-0.63 ± 0.62	-1.29 ± 0.04	-1.08 ± 0.54	p=0.135
DIO1	-0.08 ± 0.23	-0.09 ± 0.15	-0.23 ± 0.02	-0.42 ± 0.23 (*)	p=0.002
PEPCK	-0.49 ± 0.30	-0.45 ± 0.44	-0.97 ± 0.55	-0.95 ± 0.11 (*)	p=0.006