Note

Increased Expression of Hepatic Organic Cation Transporter 1 and Hepatic Distribution of Metformin in High-fat Diet-induced Obese Mice

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Summary: Although the effect of obesity on drug disposition remains an important issue for clinicians, little is known about the effects of obesity on organic cation transporter 1 (OCT1) expression and activity. Here, we show that hepatic OCT1 expression was higher in mice fed a high-fat (HF) diet for 19 weeks compared with mice fed a control diet. Since HF diet-induced obese mice exhibited elevation of plasma proinflammatory cytokines, leptin, and insulin levels, we evaluated the effect of leptin, insulin, and tumor necrosis factor-alpha (TNF-α) on OCT1 mRNA expression in HepG2 cells. Both leptin and insulin significantly increased OCT1 mRNA expression in HepG2 cells, but TNF-α did not. This finding was consistent with in vivo results. Using the OCT1 substrate metformin, we further measured the extent of hepatic uptake of metformin in obese and lean mice using the ratio of hepatic concentration to plasma concentration of metformin at 1 h after administration. The hepatic uptake of metformin was significantly higher in mice fed a HF diet compared with lean mice. In conclusion, our results suggest, at least in part, that obesity might have an effect on the absorption or distribution pharmacokinetics of metformin through an increase in hepatic OCT1 expression.

Keywords: OCT; obesity; metformin; hepatic transport; drug distribution; leptin; insulin

Interindividual variation in organic cation transporter 1 (OCT1; solute carrier (SLC)22A1) may be important in the pharmacokinetics and clinical effects of substrate drugs.1–3) In humans, OCT1 is expressed mainly in the liver and mediates the first step in the uptake of many cationic drugs across the sinusoidal membrane into hepatocytes. Because OCT1 is crucial in the disposition of cationic drugs, the identification of factors regulating OCT1 would be helpful to clinicians. In addition to the interindividual variation caused by genetic polymorphisms,2–4) the effects of environmental and/or endogenous factors on the activity of drug transporters are also important. However, information about the effects of non-genetic factors on OCT1 activity is limited, although certain pathological changes are known to regulate OCT1 expression and/or activity.5–7) The effect of obesity on the pharmacokinetics of drugs is an important issue for public health, particularly because obesity is now a major worldwide epidemic problem. However, studies on the effect of obesity on drug transporters, particularly OCT1, remain noticeably lacking in the literature. In general, the pathophysiological changes that occur in most obese individuals are characterized by hormonal changes including hyperinsulinemia, increases in adipose mass and the plasma level of leptin, fatty liver, and increases in cytokine levels; the degree to which these occur appears to be proportional to the extent of obesity.16–17) However, there appear to be no studies characterizing the effects of obesity on OCT1 expression in the liver.
Against this background, the purpose of this study was to evaluate the effects of diet-induced obesity (DIO) on the hepatic expression of OCT1 and the hepatic uptake of metformin, an OCT1 substrate, using a mouse model of high-fat (HF) DIO and to investigate the mechanisms underlying the increased hepatic expression of OCT1 in HF-DIO.

Methods

Animals and treatment: Six-week-old male C57BL/6 mice (Japan SLC Inc., Shizuoka, Japan; n = 44) were housed individually and were fed either a HF diet (modified AIN-76A diet with 40% beef tallow; Dyets, Bethlehem, PA; obese group) to produce DIO or a matched control AIN-76A diet (Dyets; lean group) for 19 weeks ad libitum as previously described.

After 19 weeks of HF or control diet feeding, mice were randomly divided into six different diet and treatment groups: (1) lean mice treated with intravenous saline (n = 4), (2) lean mice treated with intravenous metformin (Sigma-Aldrich, St. Louis, MO; n = 6), (3) lean mice treated with intraperitoneal metformin (n = 10), (4) obese mice treated with intravenous saline (n = 4), (5) obese mice treated with intravenous metformin (n = 6), and (6) obese mice treated with intraperitoneal metformin (n = 14). Since metformin is rapidly absorbed and distributed to the intestine and liver, blood was collected under i.p. secobarbital (50 mg/kg) anesthesia 1 h after intravenous metformin (50 mg/kg) or intraperitoneal metformin (150 mg/kg) administration. Simultaneously, livers were quickly removed and kept at −80°C until analysis. Plasma leptin concentration was measured using commercially available immunoassay kits (R&D Systems, Minneapolis, MN).

The concentrations of glucose, insulin, total cholesterol, triglycerides, aspartate aminotransferase in plasma were measured by commercial kits (Asan Co., Seoul, South Korea). Care of the animals and all procedures were approved by the Institutional Animal Care and Use Committee of Inha University, Incheon, Korea.

Determination of metformin concentration:

Aliquots of liver tissue were homogenized in lysis buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM Na3VO4, 1 mM dithiothreitol, 1 mM NaF, 0.8 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A, 14 μM E-64 and 1 mM AEBSF) using a Teflon homogenizer and cell membrane disruptor. Tissue extracts were used for the determination of metformin concentration and immunoblot analysis. The extracts or plasma mixed with two volumes of acetonitrile were centrifuged at 16,000 g for 10 min at 4 °C. Ten microliters of supernatant was injected directly into a high-performance liquid chromatography (HPLC) system (Shiseido Co. Ltd., Tokyo, Japan) with a Shodex ODP2 HP 2D column (2 mm × 150 mm; Showa Denko K.K., Kawasaki, Japan). The mobile phase containing 10 mM potassium phosphate (dibasic:monobasic = 1:1) in water:acetonitrile (47.8:52.2) was eluted at 0.2 mL/min and the UV detector was set at 235 nm. The determinations exhibited linearity in the range 0.05–50 μg/mL.

Determination of hepatic OCT1 expression and intracellular metformin concentration: To determine the hepatic expression of OCT1 protein according to diet, 25 μg of total protein extracts was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes and then blots were probed with a polyclonal antibody specific to the OCT1 protein (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) after blocking of membranes with Tris-buffered saline containing 5% non-fat milk and 0.1% Tween 20.

To carry out semiquantitative RT-PCR, total RNAs (3 μg) were reverse transcribed for 10 min at 25°C, 120 min at 37°C and 5 min at 85°C in the presence of random primer and MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA). To amplify the 187-bp and 198-bp fragments specific to the mouse and human OCT1 cDNA, respectively, thirty cycles of PCR reactions (denaturation, annealing and elongation at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s, respectively) were performed (Supplementary material 1). GAPDH was used for the normalization of the loading of total RNA. The intensities of target bands in Western blot and semiquantitative RT-PCR experiments were quantitatively using image analysis software Bio1D (Vilber Lourmet, France).

To determine the intracellular uptake of metformin, cells (3.5 × 105) were treated with vehicle, TNF-α (R&D Systems), leptin (R&D Systems) or insulin (Sigma-Aldrich) after 24 h attachment followed by incubation of cells with buffer (145 mM NaCl, 3 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2, 5 mM D-glucose, and 5 mM HEPES, pH 7.4) containing vehicle or metformin (300 μM) for 30 min. Cells were washed with incubation buffer three times, and then collected with incubation buffer. Collected cells were resuspended with 0.1% formic acid containing ranitidine as an internal standard and disrupted by a membrane disruptor. Samples were centrifuged at 16,000 g for 10 min and supernatants (2 μL) were injected into an HPLC system interfaced to an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada) equipped with a Turbolonspray source. The mobile phase containing 0.1% formic acid in water and 0.1% formic acid in acetonitrile (90:10) was eluted at 0.7 mL/min through an Imtakt C8 column (3 μm, 2.1 mm × 75 mm; Imtakt, Philadelphia, PA) at 40°C. Positive ion electrospray mass spectrometry was used to detect metformin and its internal standard. The source temperature was set to 600°C. The ion spray voltage and en-
trance potential were maintained at 5300 V and 10 V, respectively. Analytes were detected using multiple reaction monitoring (MRM) and the optimized collision energy was 27 V for metformin.

**Cell culture and treatment:** HepG2 cells were grown in Dulbecco's modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 100 U/mL penicillin-streptomycin (Gibco, Grand Island, NY) in a humid atmosphere containing 5% CO2 at 37°C. For the determination of OCT1 mRNA expression, after attachment for 24 h of cells in 60-mm dishes at a density of 1 × 10⁶ cells per well, cells were treated with vehicle, TNF-α, leptin or insulin for an additional 24 h.

**Statistical analysis:** Data are presented as the mean ± standard error of the mean except when stated otherwise, and p<0.05 was accepted as statistically significant. The unpaired t-test, Mann-Whitney U test or Kruskal-Wallis test with Dunn’s comparison was appropriately used to compare metformin concentrations or OCT1 expression levels using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA).

**Results**

Effects of HF diet on the hepatic expression of OCT1: To test whether DIO might increase the production of hepatic OCT1 in laboratory animals, we assessed mice fed a HF diet for 19 weeks. The body weight (47.4 ± 0.6 g; p < 0.0001) and the insulin (2.66 ± 0.46 ng/mL; p = 0.0002), leptin (6.94 ± 0.78 ng/mL; p < 0.0001), and total cholesterol levels (212.2 ± 5.50 mg/dL; p < 0.0001) of obese mice were significantly higher than those of the lean control mice (33.6 ± 0.7 g, 0.75 ± 0.18 ng/mL, 1.13 ± 0.55 ng/mL and 112.2 ± 5.21 mg/dL, respectively). In combination with elevated blood alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in HF-DIO mice (372.1 ± 63.99 and 232.7 ± 29.33 versus 59.7 ± 9.76 and 117.6 ± 12.54 in lean mice, respectively), these results imply that a HF diet for 19 weeks induces hyperleptinemia, hyperinsulinemia, hypercholesterolemia and hepatic inflammation. The levels of plasma triglyceride and glucose in the obese mice were not significantly different to those of lean mice.

The level of hepatic OCT1 mRNA expression in the HF-DIO mice was significantly higher (~2.2 fold) when compared with their lean counterparts (Fig. 1A, p = 0.007). Consistent with mRNA expression, the hepatic OCT1 protein expression of HF-DIO mice was also significantly higher (~1.6 fold) than that of lean mice (Fig. 1B, p = 0.0152).

**Effects of TNF-α, leptin and insulin on OCT1 mRNA expression and intracellular uptake of metformin:** As expected, obese mice fed with the HF diet showed increased plasma leptin and insulin. In addition, although we did not determine the plasma cytokine levels, it is well known that obesity induces an increase in plasma pro-inflammatory cytokines, such as TNF-α. Thus, we evaluated the effects of TNF-α (1, 2, or 5 ng/mL), leptin (1, 10, or 100 nM) and insulin (1, 10, or 100 nM) on the expression of OCT1 mRNA and the intracellular uptake of metformin in HepG2 cells. Treatment of cells with leptin or insulin significantly increased the OCT1 mRNA expression (Fig. 2), whereas TNF-α treatment slightly but not significantly increased OCT1 mRNA expression. Therefore, the increased expression of hepatic OCT1 in HF-DIO mice may be caused by elevation of plasma insulin, leptin and cholesterol. As a result of intracellular uptake, the intracellular concentration of metformin was significantly higher in leptin-treat-

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![Fig. 1. Expression of hepatic OCT1 mRNA and protein in lean and HF diet-induced obese mice](image)

(A) Representative bands of OCT1 mRNA. Quantitative results are presented in the graph (n = 7). ***p < 0.001 vs. lean group. (B) Representative blot of hepatic OCT1 protein. Quantitative results are presented in the graph (n = 6). **p < 0.01 vs. lean group. L and O indicate lean and obese groups, respectively.
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Fig. 2. OCT1 mRNA analysis in HepG2 cells treated with TNF-α, leptin or insulin
HepG2 cells were treated with vehicle (Con), TNF-α (1–5 ng/mL), leptin (1–100 nM) or insulin (1–100 nM) for 24 h. Quantitative data from tetraplicate experiments are presented in the graph. *p < 0.05 vs. vehicle control; Kruskal-Wallis test with Dunn’s comparison.

Fig. 3. Comparison of plasma and hepatic metformin concentration in lean and obese mice treated with intraperitoneal (150 mg/kg) or intravenous (50 mg/kg) metformin. We collected tissues and plasma 1 h after administration because metformin may completely distribute throughout tissues within 1 h.9 In addition, to reduce the large initial inter-individual variation, we set this time point to be in the early elimination phase, as previously described.19 As shown in Figure 3, we observed that the plasma and hepatic concentrations of metformin were significantly higher in the obese group compared with the lean group. This was observed for both routes of administration. In addition, when we calculated the ratio between hepatic and plasma concentrations after intraperitoneal administration of metformin, the ratio of the obese group (0.053 ± 0.005, p = 0.0038) was significantly higher than that of the lean group (0.028 ± 0.004). For intravenous administration of metformin, the ratio of the obese group (0.029 ± 0.004) was also significantly higher than that of the lean group (0.019 ± 0.002, p = 0.0317). The higher plasma concentration of metformin in obese mice might be caused by the larger dose of metformin in obese animals (dose per body weight). These results indicate that the hepatic uptake of metformin was increased by HF-DIO, which might result, at least in part, from the induction of OCT1 expression.

Discussion
To date, the regulation of OCT1 has been observed in various pathological conditions.6,10,11 It has been report-
ed that agonists of the peroxisome proliferator agonist receptor (PPAR) activate the PPAR-response element in the promoter region of the Slc22a1 gene and that the pregnane X receptor ligand increases the transcriptional activity of Slc22a1; however, further characterization of the regulation of Slc22a1 is needed.\textsuperscript{11,13} In contrast to PPAR agonists, pro-inflammatory cytokines reduce the expression of various hepatic transporters, including OCT1.\textsuperscript{11,13} Serum levels of C-reactive protein, a sensitive marker of systemic inflammation, are higher in obese people than in people of normal weight.\textsuperscript{10} Furthermore, the levels of pro-inflammatory cytokines, such as interleukin 6 and tumor necrosis factor-\(\alpha\) are significantly higher in obese subjects than in lean subjects.\textsuperscript{15–17} Therefore, it is probable that OCT1 expression is reduced by the increased pro-inflammatory cytokines that occur in obesity. However, our results do not support this. Together with increases in pro-inflammatory cytokines in obesity, there are increases in insulin levels and modulations of adipokines, such as leptin and adiponectin. It has been demonstrated that experimental insulin-deficient diabetes decreased the expression of renal OCT1 protein in rats. This decrease in OCT1 was partially recovered by insulin therapy, implying that insulin or glucose may be a regulator of OCT1.\textsuperscript{7} Additionally, we observed hypercholesterolemia in our obesity model. It has been report-
ed that treatment of HepG2 cells with cholesterol increases OCT1 mRNA expression.\textsuperscript{20} Therefore, it is difficult to determine the result of combined effects of various factors that arise from obesity on the regulation of hepatic OCT1 expression. Our \textit{in vivo} results make it necessary to further investigate the factor(s) responsible for the increase of hepatic OCT1 expression in HF-DIO and its clinical implications.

Our mice fed with a HF diet did not show hyperglycemia. Grover et al. reported that type 1 diabetic rats showed a decrease in renal OCT1 expression, which was recovered by insulin therapy.\textsuperscript{3} Therefore, insulin, but not plasma glucose, may be a factor regulating hepatic OCT1 expression. Obesity caused numerous pathophysiological changes including the elevation of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-\(\alpha\)) and interleukin-6 (IL-6). Recently, it was reported that both TNF-\(\alpha\) and IL-6 were found to decrease OCT1 mRNA expression in human primary hepatocytes.\textsuperscript{15} The discrepancy between the previous findings and our current data may result from the different cellular model and treatment conditions used. However, the combined effects of the pathophysiological changes observed in obesity might cause an increase in the hepatic expression of OCT1. To support this, our data suggested that leptin and insulin may be inducers of hepatic OCT1. Although we failed to find that insulin significantly increased the intracellular uptake of metformin, leptin significantly increased both OCT1 mRNA expression and intracellular uptake of metformin in HepG2 cells. When combined, our results and previous observations (increase of OCT1 by insulin and cholesterol) indicate that leptin and/or insulin and cholesterol may be factors responsible for the increased hepatic distribution of metformin in HF-DIO mice.

We did not evaluate OCT1 and OCT2 expression in the kidneys of obese animals, which is a limitation of our study. Metformin is mainly cleared through renal elimination. Since OCT2 may be important to the elimination of metformin,\textsuperscript{21} if it occurs the modulation of renal or hepatic OCT2 in obesity would also be important to the pharmacokinetic alteration of metformin in obesity. Our study research focused on the alteration of OCT1 and hepatic distribution of metformin in obesity; therefore, further research should be done to elucidate this issue using diet-induced obese animals.

We did not detect a significant pharmacodynamic difference in the activation of hepatic 5’-adenosine monophosphate-activated protein kinase (AMPK) because of large interindividual variation (data not shown). The phosphorylation of hepatic AMPK is very sensitively regulated by feeding status.\textsuperscript{22} Because we did not restrict the feeding of mice during the experiment, the large interindivid-
ual variability of AMPK phosphorylation caused by uncontrolled feeding may have interfered with the detection of a significant change.

In conclusion, to our knowledge, the regulation of OCT1 by DIO has not been observed previously. This study, has shown for the first time that HF-DIO increases the expression of hepatic OCT1 and the hepatic distribution of metformin. The increased levels of insulin and leptin may contribute to the induction of hepatic OCT1 expression, at least in part. Because our results did not resolve the effects of OCT2 and the pharmacodynamic differences caused by OCT1 induction in the obese condition, this issue requires further investigation to clarify the clinical implications.

\textbf{References}


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