SLCO4C1 Transporter Eliminates Uremic Toxins and Attenuates Hypertension and Renal Inflammation

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ABSTRACT

Hypertension in patients with chronic kidney disease (CKD) strongly associates with cardiovascular events. Among patients with CKD, reducing the accumulation of uremic toxins may protect against the development of hypertension and progression of renal damage, but there are no established therapies to accomplish this. Here, overexpression of human kidney-specific organic anion transporter SLCO4C1 in rat kidney reduced hypertension, cardiomegaly, and inflammation in the setting of renal failure. In addition, SLCO4C1 overexpression decreased plasma levels of the uremic toxins guanidino succinate, asymmetric dimethylarginine, and the newly identified trans-aconitate. We found that xenobiotic responsive element core motifs regulate SLCO4C1 transcription, and various statins, which act as inducers of nuclear aryl hydrocarbon receptors, upregulate SLCO4C1 transcription. Pravastatin, which is cardioprotective, increased the clearance of asymmetric dimethylarginine and trans-aconitate in renal failure. These data suggest that drugs that upregulate SLCO4C1 may have therapeutic potential for patients with CKD.


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All individuals with an estimated GFR (eGFR) <60 ml/min per 1.73 m² are defined as having chronic kidney disease (CKD). The prevalence of CKD is now estimated at approximately 10% of the population and will progress to ESRD. In patients with CKD, the accumulation of uremic toxins causes difficulty in controlling BP, impairs renal function, and worsens prognosis. So far, more than 110 organic compounds have been identified as uremic toxins. Among these, guanidino compounds, including guanidino succinate (GSA) and asymmetric dimethylarginine (ADMA), are increased in patients with CKD and correlate with prognosis. In particular, ADMA, an inhibitor of nitric oxide synthase, is implicated in hypertension, renal damage, cardiac hypertrophy, and cardiovascular events. Currently, administration of the oral adsorbent AST-120 is the only therapy to remove uremic toxins in patients with CKD and diabetic nephropathy. Although AST-120 removes indoxyl sulfate, other compounds are not eliminated. Thus, a new approach that addresses this problem is urgently needed.

Recently, we isolated a human kidney-specific organic anion transporting polypeptide (OATP), termed SLCO4C1, and functionally characterized it as a digoxin transporter. The OATP family is involved in the membrane transport of bile acids, conjugated steroids, thyroid hormone, eicosanoids, peptides, cardiac glycosides (digoxin, digitoxin, and ouabain), and numerous drugs. Among these, in the kidney, SLCO4C1 might be a first step of transport pathway of digoxin and various compounds into urine. In renal failure, basolateral SLCO4C1 expression was decreased; however, the expression level of MDR1, a member of the ATP-binding cassette transporter family that mediates the tubular secretion of digoxin at the apical membrane of the proximal tubule cell, was not changed. This reduction of SLCO4C1 in the proximal tubules may be one of the mechanisms of impaired urinary excretion of digoxin and drugs in renal failure. In humans, SLCO4C1 is the only organic anion transporter in the kidney, whereas, in rodent kidney, several oatps exist at the basolateral and apical membrane of the proximal. This species diversity of the OATP family subtypes and the multiple locations in proximal tubules make it difficult to extrapolate from experimental studies of rodents to humans. To overcome this issues, here, we generated a transgenic (TG) rat harboring human SLCO4C1 in rat kidney and clarified physiologic and pathophysiologic roles of human SLCO4C1.

RESULTS

Generation of TG Rat Harboring Human SLCO4C1 in the Kidney

TG rat harboring human SLCO4C1 in the kidney was generated using the proximal tubule–specific promoter (Figure 1A and B). In addition, to avoid unusual mRNA splicing during overexpression, we mutated three atypical splicing donor-adaptor sites in the coding region of SLCO4C1 without changing the amino acids (Figure 1A). As a result, the human SLCO4C1 mRNA was exclusively expressed in the kidney, especially in the proximal tubules of TG rats (Figure 1C). Immunohistochemical analysis also revealed that human SLCO4C1 protein was strongly detected at the basolateral side of the proximal tubules (Figure 1D).

When renal mass was reduced by five-sixths nephrectomy (Nx), BP was significantly decreased in TG(+)Nx rats compared with non-TG littermate [TG(−)Nx] rats (Figure 2A). In addition, to avoid unusual mRNA splicing during overexpression, we mutated three atypical splicing donor-adaptor sites in the coding region of SLCO4C1 without changing the amino acids (Figure 1A). As a result, the human SLCO4C1 mRNA was exclusively expressed in the kidney, especially in the proximal tubules of TG rats (Figure 1C). Immunohistochemical analysis also revealed that human SLCO4C1 protein was strongly detected at the basolateral side of the proximal tubules (Figure 1D).

When renal mass was reduced by five-sixths nephrectomy (Nx), BP was significantly decreased in TG(+)Nx rats compared with non-TG littermate [TG(−)Nx] rats (Figure 2A). This BP reduction was seen in two independently generated lines. In TG(+)Nx rats, cardiac hypertrophy was also significantly reduced (Figure 2B).
The survival rate of TG(+)Nx rats was slightly increased from that of TG(−)Nx rats, but the results did not reach statistical significance (Supplemental Figure 1C). In patients with CKD, renal inflammation is also a risk factor of renal damage and morbidity and mortality.12 Immunohistochemically, mononuclear cell infiltration stained with the macrophage marker CD68 was strongly detected in TG(−)Nx rat kidneys (Figure 2C). Conversely, TG(+)Nx kidneys demonstrated less infiltration of macrophage (Figure 2C). These data indicate that expression of human SLCO4C1 in rat kidneys ameliorated not only hypertension but also inflammation in renal failure.

Elimination of Uremic Toxins in TG(+) Rats

To understand the mechanism by which SLCO4C1 exerted anti-hypertensive and anti-inflammation effects, we performed comprehensive qualitative metabolome analysis.13 Blood and urine specimens were measured by capillary electrophoresis mass spectrometry (CE-MS) and HPLC, and 188 anions and 298 cations were identified (Supplemental Tables 1 through 4). Among these, we focused on 21 compounds for which concentration was significantly changed after Nx (Supplemental Figure 2). As a result, the plasma levels of creatinine and indoxyl sulfate were increased 3 wk after Nx as previously reported,4 but the concentrations of these compounds were not different between TG(+)Nx and TG(−)Nx rats 3 wk after Nx (Figure 3, A and B). Conversely, although the plasma concentration of ADMA, GSA, and trans-aconitate were significantly increased 3 wk after Nx, the increments were significantly decreased in TG(+)Nx rats compared with TG(−)Nx rats (Figure 3, C through E). These data suggest the facilitation of the excretion of uremic toxins in TG(+) rats.

To exclude the possibility of the compensative or nonspecific effects by overexpression of SLCO4C1 in the kidney, we performed microarray analysis. As a result, there was NS difference in the expression levels of other rat transporters (slco4c1, oatp1, oatp3, oatp5, abcb11, mrp2, mdr1, and mlc1).

The serum ADMA level is controlled by two pathways: (1) Enzymatic degradation by dimethylarginine dimethylaminohydrolase (DDAH) and (2) urinary excretion.14 In TG(+)Nx rats, the DDAH1 mRNA level was not different between TG(+)Nx and TG(−)Nx rats, and the DDAH2 mRNA level in TG(+)Nx rats was decreased compared with TG(−)Nx rats (Figure 3F), suggesting that the decrease of ADMA in TG(+)Nx rats was not dependent on facilitating enzymatic degradation. In addition, neither the plasma level of citrulline (Figure 3G), produced from ADMA by DDAHs, nor the mRNA level of protein arginine N-methyltransferase that generates ADMA from arginine was different between TG(−)Nx and TG(+)Nx rats. Because GSA excretion had not completely correlated with creatinine clearance,15 these data further suggest that the overexpression of SLCO4C1 at the proximal tubule facilitates guanidino compound excretion in renal failure.

Trans-aconitate is a competitive inhibitor of aconitase.16 Aconitase is a key enzyme in catalyzing citrate to isocitrate via cis-aconitate in the TCA cycle, and the accumulation of trans-aconitate inhibits TCA cycle and respiration in tissues.16 The retention compounds that are biologically/biochemically active and responsive for the uremic syndrome are called uremic toxins.4 It is widely known that he accumulation of guanidino compounds (including ADMA and GSA) and several uremic toxins generate oxidative stress, and it causes further renal
damage in patients with CKD\(^2\); however, the existence in mammals, biologic effects, and the precise role of trans-aconitate in renal failure have not been clarified. When trans-aconitate was administered to rats intraperitoneally, the BP of injected rats was immediately elevated compared with controls (Figure 3H). This increase of BP was cancelled when trans-aconitate was injected into TG(+) rats compared with TG(−) rats, further suggesting the excretion through SLCO4C1 (Supplemental Figure 1D). In addition, trans-aconitate significantly induced superoxide production in human kidney proximal tubule cells (Figure 3I).

To confirm further that not only ADMA and GSA but also trans-aconitate exists in humans and the concentration is increased in accordance with CKD progression, we performed CE-MS analysis of 41 patients with CKD at various stage. The plasma level of trans-aconitate was significantly correlated with the increase of plasma creatinine, and that inversely correlated with the eGFR similar to ADMA and GSA (Figure 4). Because the plasma level of trans-aconitate in patients without CKD is low, these data suggest that trans-aconitate can be a new uremic toxin, and a newly identified biomarker for predicting the onset of renal damage and, thus, the elimination of trans-aconitate plays a beneficial role in CKD.

**Functional Analysis of SLCO4C1 Promoter and Its Modulation by Statins**

We assumed that enhancement of SLCO4C1 in the kidney may facilitate the excretion of uremic toxins and thereby ameliorate the symptoms of CKD. In this scenario, drugs that upregulate SLCO4C1 in the kidney may facilitate excretion of uremic toxins and reduce renal inflammation, decelerating progression of renal damage and entry of hemodialysis. To address this, we isolated the promoter region of human SLCO4C1. Human SLCO4C1 promoter region has a predominant transcription start site located 164 bp upstream of the ATG codon (Figure 5A). Potential cis-acting motifs for GATA-1, hepatocyte nuclear factor (HNF)-3α, CCAAT/enhancer-binding protein (C/EBP)α, C/EBPβ, cAMP response element-binding protein (CREB), and peroxisome proliferator-activated receptor α were found. We also identified tandem xenobiotic-responsive element (XRE) motifs containing the substitution-intolerant core sequence 5′-CACGC-3′ at position −126 (GGCAGCCAGCGCC). That sequence is generally recognized by AhR and AhR nuclear translocator heterodimer,\(^18\) although the flanking sequences are not typical compared with cyp1a1 XRE motifs\(^19,20\) (Supplemental Figure 3D). AhR binds “classical” ligands such as the environmental pollutants halogenated aromatic hydrocarbons (e.g., dioxin, benzo[a]pyrene, 3-methylcholanthrene [3-MC]).\(^21\)

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**Figure 3.** Metabolome analysis and characterization of uremic toxins are shown. (A through E and G) The plasma concentration of creatinine (A), indoxyl sulfate (B), ADMA (C), GSA (D), trans-aconitate (E), and citrulline (G) before and 3 wk after five-sixths Nx (n = 4 to 5 per group). (F) The mRNA expression level of DDAH1 and DDAH2 in the kidney 3 wk after five-sixths Nx (n = 5 per group). (H) BP after intraperitoneal injection of trans-aconitate (400 mg/kg; n = 5 per group). (I) Trans-aconitate–induced superoxide production in HK-2 cells. *P < 0.05.
Human SLCO4C1 promoter activity was increased 1.49-fold (2064) and 1.68-fold (129) by 3-MC compared with controls (Figure 5B). The 129 construct exhibited the highest activity, and this segment contained XRE core motifs. Because AhR can also bind to a structurally divergent range of chemicals,21 we next screened various compounds. The hepatic hydroxymethyl glutaryl–CoA reductase inhibitor (statin) fluvastatin (2.3-fold at 10 μM) and pravastatin (1.3-fold at 30 μM) and atypical AhR ligand flutamide (1.4-fold at 10 μM) upregulated the SLCO4C1 promoter activity (Figure 5C). Because of the comparable magnitude to 3-MC and its clinical availability, we further focused on statins. Deletion experiments showed that all constructs exerted potent promoter activation, but removal of the XRE core segment or mutation in the XRE core motifs abolished the response to fluvastatin (Figure 5D). Because there are various clinical reports on renoprotective effects of statins,22 we further examined various statins on human SLCO4C1 transcription. Simvastatin, lovastatin, cerivastatin, mevastatin, atorvastatin, rosuvastatin, and pitavastatin upregulated SLCO4C1 transcription (Figure 5F).

Next, we determined the ligand-dependent recruitment of the AhR–XRE system by chromatin immunoprecipitation (ChIP) assay. Application of the antibody against AhR resulted in a positive band for both 3-MC and fluvastatin (Figure 5E, top). In addition, the nuclear recruitment of AhR protein was further confirmed by Western blotting with a strong band in the nuclear extract by 3-MC and fluvastatin (Figure 5E, bottom). These data suggested that statins regulate SLCO4C1 transcription through the AhR–XRE system.

Statins Increase Tubular Uremic Toxin Excretion
On the basis of our results, we next examined the effect of statins in renal failure. In human kidney proximal cells, application of fluvastatin and pravastatin significantly potentiated the SLCO4C1 mRNA by 1.72- and 1.73-fold, respectively (Figure 6A). The uptake of thyroid hormone T3, a representative ligand of SLCO4C1, was also significantly potentiated by fluvastatin and pravastatin by 1.3- and 1.4-fold, respectively (Figure 6B), suggesting the potentiation of SLCO4C1 function in the proximal tubules.

We next examined the effects of pravastatin in vivo. We and other groups reported that pravastatin reduced BP.23,24 In addition, pravastatin has been reported to modulate DDAH activity and modulate ADMA concentration.25 To avoid the effect on BP and to eliminate other pleiotropic effects of pravastatin, we administered low-dosage pravastatin to Nx Wistar rats and examined renal tubular function. After administration of pravastatin, BP was not changed but the mRNA level of rat slco4c1 was significantly increased in the kidney (Figure 7, A and B). Under this condition, the ADMA and trans-aconitate clearance were significantly increased in pravastatin-treated Nx rats without changing creatinine clearance, although the GSA clearance was not statistically significant (Figure 7, C through F). Furthermore, the mRNA level of DDAHs, protein arginine N-methyltransferases, or other transporters was not changed (data not shown). These data strongly suggested that pravastatin increased ADMA and trans-aconitate excretion in the proximal tubules. In addition, cardiac hypertrophy was decreased in the pravastatin-treated group (Figure 7G).

**DISCUSSION**
Here, we found that the plasma concentration of uremic toxins ADMA, GSA, and trans-aconitate were significantly reduced in
TG(+)Nx rats. The guanidino compounds are a large group of structural metabolites of arginine, and the concentrations of GSA and ADMA are markedly increased in renal failure.12-13 GSA accumulation causes various harmful effects, such as inhibition of platelet aggregation, hemolysis, and convulsions.26 Likewise, ADMA is the most specific endogenous compound that inhibits the TCA cycle16; however, its existence in mammals, especially in renal failure, was not previously known. Compounds that inhibit the TCA cycle are “poison.” It is also widely known that fluoroacetate is a “suicide” substrate for aconitase.

Acute fluoroacetate poisoning in humans mainly affects the central nervous system, cardiovascular system, and kidney, and the biochemical effects include TCA cycle blockade, respiratory failure, and metabolic acidosis and lactate accumulation.24 Trans-aconitate administration also increased BP and generated oxidative stresses in rats. These data suggest that the overexpression of SLCO4C1 in the renal proximal tubules in TG(+) rats causes the beneficial effect of excretion of harmful uremic toxins such as ADMA, GSA, and trans-aconitate and proposes a new approach to decrease uremic toxins and to reduce the exacerbation of renal function in patients with CKD (Figure 8).

Here we show that statins function as a nuclear receptor ligand recruiting the AhR-XRE system and upregulating SLCO4C1 tran-
Pravastatin was provided by Daiichi-Sankyo (Tokyo, Japan). Other statins were purchased from Sequoia Sciences (St. Louis, MO).

Materials

Pravastatin was provided by Daiichi-Sankyo (Tokyo, Japan). Other statins were purchased from Sequoia Sciences (St. Louis, MO).

Construction of Kidney-Specific TG Rats

The mutated coding region of human SLCO4C1 was inserted into the pGEM-sglt2–5pr-mut plasmid containing kidney-specific sglt2 promoter.
The linear purified plasmid was injected into the pronuclei of fertilized oocytes of Wistar rats. Pups were analyzed for the genomic integration by Southern blotting and by PCR amplification of tail DNA using the following primers: Forward (mouse sglt2) 5′-tccccccacttctgtttcccagtctatgt-3′ and reverse (human SLCO4C1) 5′-acgcgatctgcagaattaagcttgggctc-3′. Reverse transcriptase–PCR was carried out using the same primers that can amplify the full length of human SLCO4C1 cDNA. Resultant TG(+/H11001) rats showed normal breeding and development with no obvious phenotypic abnormalities in body weight, water and food intake, and renal functions compared with TG(+/H11002) littermates, whose genetic background is the same as that of TG(+/H11001) rats except for expression of human SLCO4C1 (Supplemental Figure 1A). All animal experiments were approved by the Tohoku University Animal Care Committee.

Immunohistochemistry

The rabbit antiserum against 107 peptides of the N-terminus of human SLCO4C1 was raised and immunopurified. Western blotting and immunohistochemistry were performed as described previously, and the quality was confirmed by peptide absorption (Supplemental Figure 1, B and D). The mouse mAb against CD68 was purchased from Serotec (Martinstried, Germany).

Figure 7. Effects of pravastatin in vivo. (A) BP in control and pravastatin-treated (0.1 mg/ml drinking water) rats after five-sixths Nx (n = 6 to 7 per group). (B) The mRNA expression of rat slco4c1 in the kidney after pravastatin administration (n = 11 per group). (C through F) Renal clearance of creatinine (C), ADMA (D), trans-aconitate (E), and GSA (F) 3 wk after five-sixths Nx (n = 5 to 7 per group). (G) Thickness of the interventricular septum (IVSTd) and left ventricular posterior wall at end-diastole (LVPWTd) before and after five-sixths Nx (n = 6 to 7 per group). *P < 0.05.

Figure 8. Uremic toxins and SLCO4C1 transporter in renal failure. ADMA is formed by protein arginine N-methyltransferase (PRMT) from arginine and degrades to citrulline by DDAH. Note that SLCO4C1 facilitates the excretion of GSA, ADMA, and trans-aconitate and that statins increase the expression and the function of SLCO4C1, resulting in reductions of the uremic toxins and BP. Trans-aconitase inhibits aconitase activity and induces reactive oxygen species (ROS). Aco, aconitase.
Nephrectomized Rat Model and BP Measurement
Five-sixths nephrectomized rats were generated as previously reported. Briefly, male TG rats were intraperitoneally anesthetized with ketamine (30 mg/kg) and xylazine (2 mg/kg) and subjected to five-sixths renal ablation. At the time of surgery, rats were prepared for telemetric monitoring of BP (Data Sciences Int., St. Paul, MN).

Echocardiogram
Rats were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) and studied with Doppler imaging by echocardiogram. The thickness of the interventricular septum and the left ventricular posterior wall at end-diastole were measured as described previously.

CE-MS Method for Metabolome Analysis
A comprehensive and quantitative analysis of charged metabolites by CE-MS was performed. Metabolites were first separated by CE on the basis of charge and size and then selectively detected using MS by monitoring over a large range of m/z values. Plasma and urine ADMA were measured by HPLC. Anionic and cationic compounds that were increased or decreased after Nx in both of the generated rat lines were nominated as statistically significant and are summarized in Supplemental Figure 2 (all analyzed CE-MS data are in Supplemental Tables 1 through 4). In the human plasma analysis, the protocols conformed to the ethical guidelines and approvals of both Tohoku University and Nagasaki University. Informed consent was obtained from each participant. The eGFR was calculated with the formula42 eGFR (ml/min per 1.73 m²) = 175 \times \text{creatinine}^{-1.154} \times \text{age}^{-0.203} \times 0.742 (\text{if female}) \times 0.741.

Measurement of Reactive Oxygen Species
The free radical formation within the human kidney proximal cell line HK-2 evoked by trans-acetanitrate (100 μM) was monitored by measurement of the changes in fluorescence resulting from the oxidation of dihydroethidium to ethidium as the increase of ethidium production (U/s) using a 505-nm dichroic mirror with the 605/55-nm band-pass filter of an IX71 microscope (Olympus, Tokyo, Japan).

Transcriptional Assay
The human SLCO4C1 promoter DNA fragments were amplified by PCR, and the amplified fragments were inserted into the pGL3 basic luciferase expression vector (Promega, Madison, WI). The point mutation of two XREs was generated by PCR. Two micrograms of plasmid construct was transfected with 0.1 μg of Renilla luciferase Reporter Vector pHRL-TK (Promega) as well as co-transfection with AhR and AhR protein-DNA complexes were immunoprecipitated using rabbit polyclonal antibody against AhR (BIOMOL, Plymouth, PA) or nonspecific antirabbit IgG. The recovered DNA was then subjected to PCR using primers that amplify regions containing the CRE elements of the human SLCO4C1 gene (forward primer 5'-AAGGGAGCTTATGGCCA-GAGACTC-3' and reverse primer 5'-TGGGCTCAAGGACCAGGGAG-3') or mouse cyp1a1 gene (forward primer 5'-CTATCTCTTAAACCCGACCCCA-3' and reverse primer 5'-CTAAGTATGTTGGAGAAAGGTTG-3'). Nuclear and cytoplasmic fraction extracts were prepared and Western blotting was performed as described previously by using antibodies against AhR, Lamin B (Santa Cruz Biotechnology, Santa Cruz, CA), and α-tubulin (Sigma-Aldrich, St. Louis, MO).

Real-Time PCR Analysis
We performed real-time PCR analysis with probe sets from Applied Biosystems (Foster City, CA).

Statistical Analysis
The data are means ± SEM. We used an unpaired t test for comparisons between two groups. For multiple comparisons, we used two-way ANOVA with repeated measures in Figures 2A, 3H, and 7A and Supplemental Figure 1D and ANOVA on rank in Supplemental Figure 3, A through C. We derived P values for Supplemental Figure 1C using log-rank test. In Figure 4, Spearman rank correlation was calculated. P < 0.05 was considered to be significant.

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DISCLOSURES
None.

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