

lifetimes. Further study is required to obtain risk stratifications for the stage 3 and 4 populations.

In the diabetic population, the prevalences of high GFR (GFR ≥ 120 ml/min/1.73 m²) and low GFR (<40 ml/min/1.73 m²) were higher than those in the population with HbA1c <6.0%, suggesting that diabetes shifts the distribution of GFR to the high and low ranges. We speculated that hyperfiltration plays a major role in this shift, and may contribute to the rapid decline in GFR in diabetic individuals. Hyperfiltration may aid the development of microalbuminuria in type 1 diabetic patients. Amin and colleagues reported a strong relationship between the risk for the development of microalbuminuria in individuals who had diabetes for five and ten years and the development of glomerular hyperfiltration in individuals who had diabetes for five years, independent of glycemic control [18].

The prevalence of CKD comorbid with other concurrent conditions in the Japanese population was similar to the corresponding prevalences in the US and Chinese populations. The prevalence of CKD was higher among hypertensive and diabetic individuals in the white US population, as previously reported [19]. The prevalence of CKD was reported to increase in hypertensive and diabetic populations in Chinese [20]. This study also supports the notion that prevalence of CKD comorbidity is higher in hypertensive and diabetic populations than in the normal population.

We previously reported that the rate of decline of GFR was more than twofold faster when the eGFR was less than 50 ml/min/1.73 m² in adults [17]. From the viewpoint of risk stratification for progression to ESKD, we estimated that 3.1% of the adult population (3.17 million) had GFR <50 ml/min/1.73 m² in 2005 (Table 3). Presence of proteinuria is a strong risk factor for ESKD and CVD. From Table 3, 2.74 million (2.7%) of the adult population have proteinuria and GFR >50 ml/min/1.73 m². Taken together, the CKD population with risk of progression to ESKD is predicted to be 5.91 million, 5.8% of the adult population in 2005.

The limitations of the present study are as follows. First, the study cohort was a proportion of the general population that participated in an annual health check program; it was not representative of the whole Japanese population. Second, the serum creatinine was not measured at a single laboratory, so the values of serum creatinine may have drifted. Third, we only measured proteinuria once. Therefore, the presence of proteinuria was confirmed, not persistent proteinuria.

In conclusion, about 13% of Japanese adult population, approximately 13.3 million people, were predicted to have CKD in 2005. From the viewpoint of risk stratification to progression to ESKD, about 5.8% of the adult population—

approximately 6 million people—who have proteinuria or GFR <50 ml/min/1.73 m² are estimated to have CKD in Japan.

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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).¹ 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.^{2,3} So far, more than 110 organic compounds have been identified as uremic toxins.⁴ Among these, guanidino compounds, including guanidino succinate (GSA) and asymmetric dimethylarginine (ADMA), are increased in patients with CKD and correlate with prognosis.^{3,5} In particular, ADMA, an inhibitor of nitric oxide synthase, is implicated in hypertension, renal damage, cardiac hypertrophy, and cardiovascular events.^{6,7} Currently, administration of the oral adsorbent AST-120 is the only therapy to remove uremic toxins in patients with CKD and diabetic nephropathy.⁸ Although AST-120 removes indoxyl sulfate, other compounds are not eliminated.⁹ 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.¹⁰ 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.¹⁰ Among these, in the kidney, SLCO4C1 might be a first step of transport pathway of digoxin and various compounds into urine.¹⁰ 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.¹⁰ This reduction of SLCO4C1 in the proximal tubules may be one of the mechanisms of impaired urinary ex-

cretion of digoxin and drugs in renal failure.¹⁰ 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.¹⁰ 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.

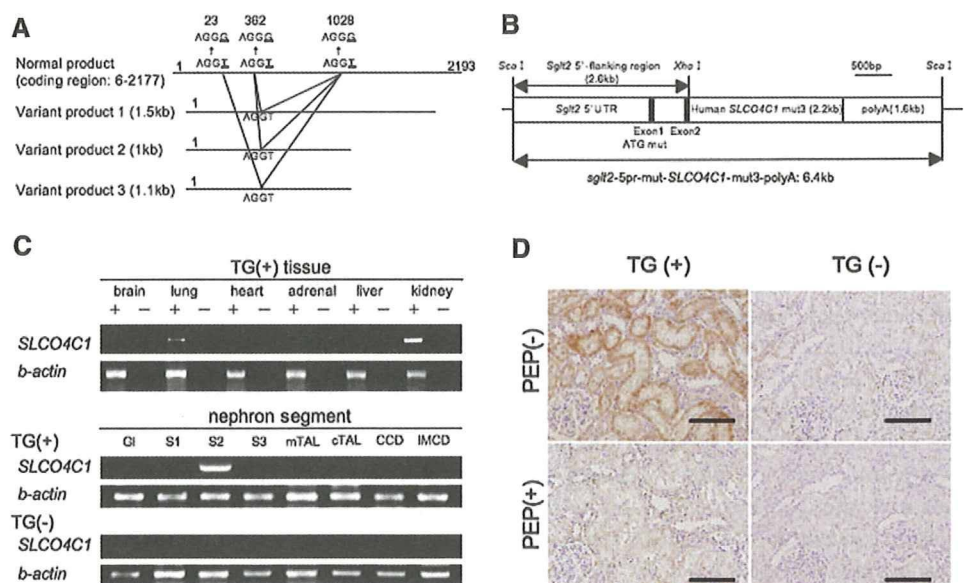
RESULTS

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 promoter¹¹ (Figure 1, A 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). This BP reduction was seen in two independently generated lines. In TG(+)Nx rats, cardiac hypertrophy was also significantly reduced (Figure 2B).

Figure 1. Characterization of human SLCO4C1 TG rats is shown. (A) Three different smaller sizes of mRNA by alternative splicing were found and mutated to avoid unusual splicing (AGGT to AGGG). (B) The mutated human SLCO4C1 cDNA was inserted into a plasmid under the proximal tubule-specific promoter. (C) Expression of human SLCO4C1 in rat organs and microdissected renal tubules examined by reverse transcriptase-PCR. Gl, glomerulus; S1, proximal tubule S1 segment; S2, proximal tubule S2 segment; S3, proximal tubule S3 segment; mTAL, medullary thick ascending limb; cTAL, cortical thick ascending limb; CCD, cortical collecting duct; IMCD, inner medulla collecting duct. (D) Immunohistochemical analysis. The human SLCO4C1 immunostains were abolished by peptide absorption. Bars = 100 μ m.



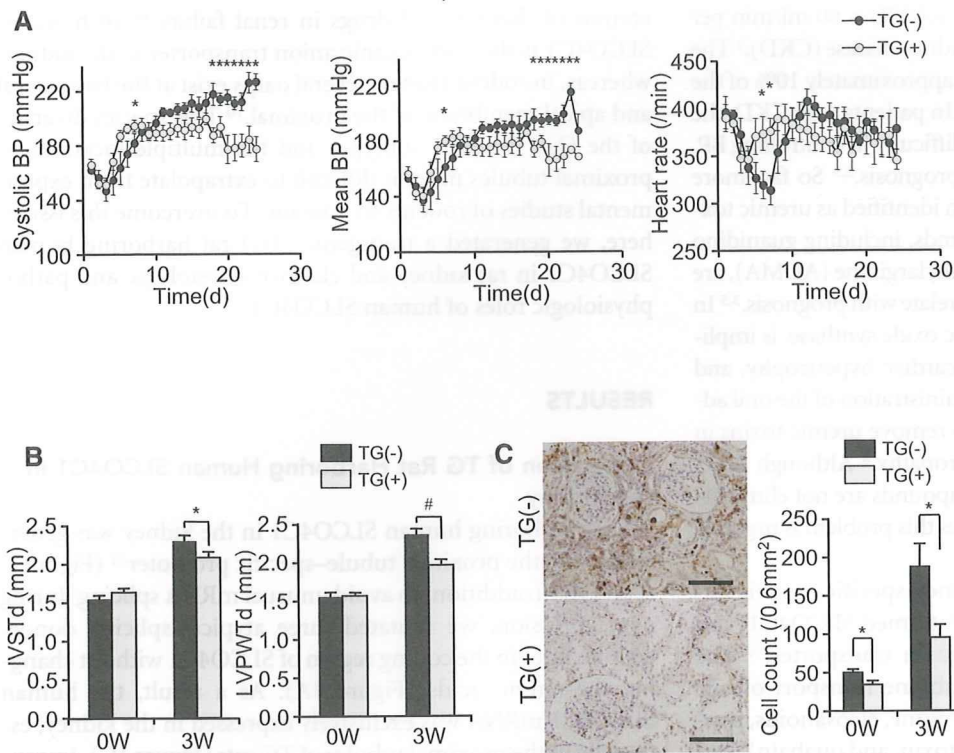


Figure 2. Phenotype of human SLCO4C1 TG rats. (A) BP and heart rate of TG(-)Nx and TG(+)Nx rats. **P* < 0.05 versus TG(-)Nx rats (*n* = 4 to 6 per group). (B) Thickness of the interventricular septum (IVSTd) and left ventricular posterior wall at end-diastole (LVPWTd) were measured by echocardiogram before and 3 wk after five-sixths Nx. **P* < 0.05; #*P* < 0.01 (*n* = 4 to 9 per group). (C) CD68 staining in the rat kidney before and 3 wk after five-sixths Nx. CD68⁺ cell number counts were performed before and 3 wk after five-sixths Nx. **P* < 0.05 versus TG(-) rats (*n* = 6 to 9 per group). Bars = 100 μm.

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 quantitative 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 the accumulation of guanidino compounds (including ADMA and GSA) and several uremic toxins generate oxidative stress, and it causes further renal

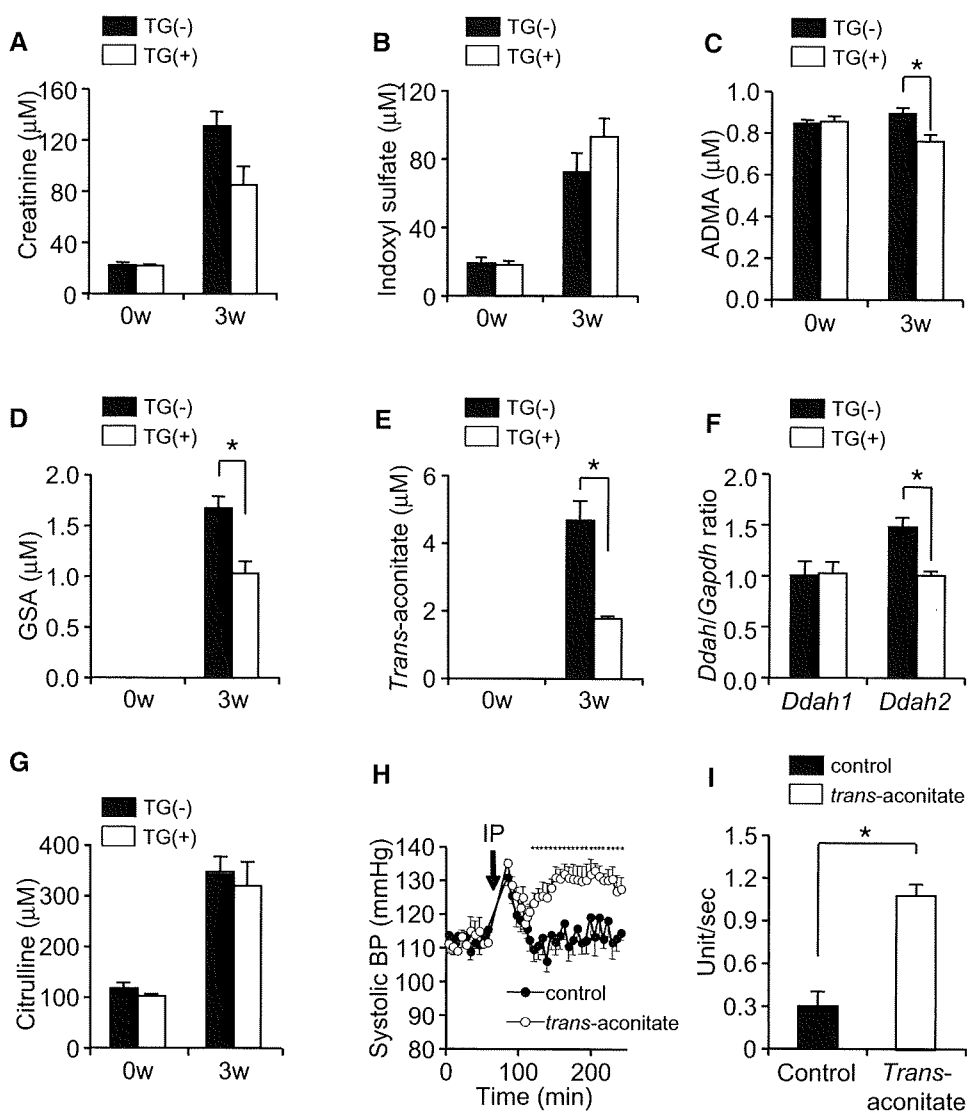


Figure 3. Metabolome analysis and characterization of uremic toxins are shown. (A through E and G) The plasma concentration of creatinine (A), indoxyl sulfate (B), ADMA (C), GSA (D), *trans*-aconitate (E), and citrulline (G) before and 3 wk after five-sixths Nx ($n = 4$ to 5 per group). (F) The mRNA 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. * $P < 0.05$.

damage in patients with CKD¹⁷; 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)-3 α , CCAAT/enhancer-binding protein (C/EBP) α , C/EBP β , 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 (GGCAGCCCACGCCG). 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

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 μ M) and pravastatin (1.3-fold at 30 μ M) and atypical AhR ligand flutamide (1.4-fold at 10 μ M) 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, itavastatin, 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.

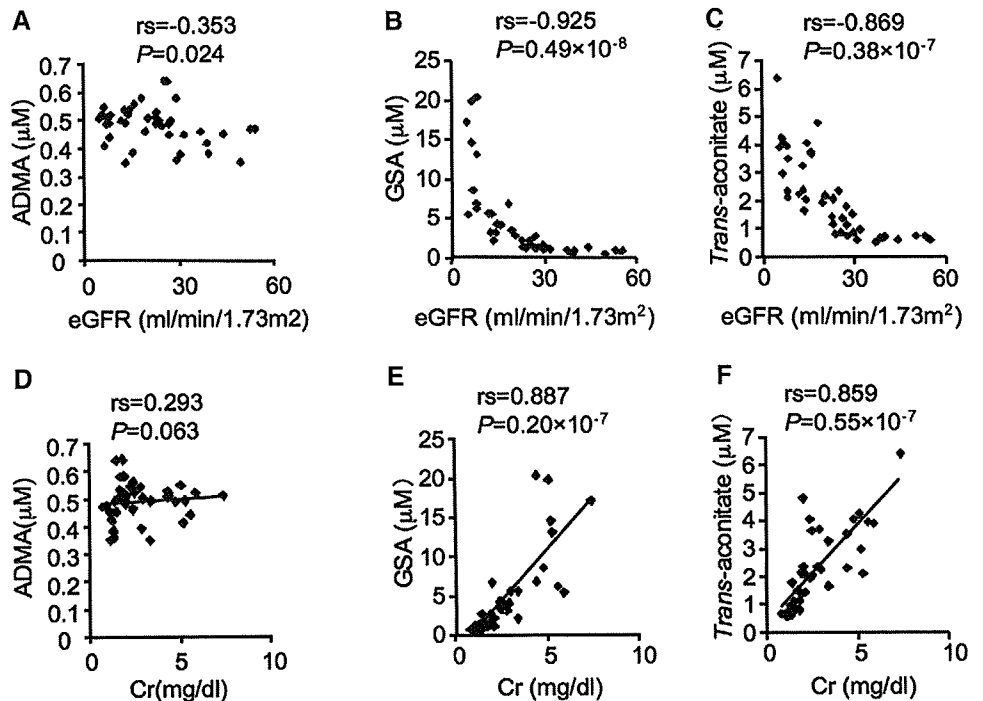


Figure 4. Relation between uremic toxins and eGFR as well as plasma creatinine in 41 patients with CKD is shown. (A through C) Correlations between eGFR and the plasma ADMA (A), GSA (B), and *trans*-aconitate (C) in patients with CKD. (D through F) Concentrations between plasma creatinine (Cr) and plasma ADMA (D), GSA (E), and *trans*-aconitate (F).

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 *slo4c1* 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

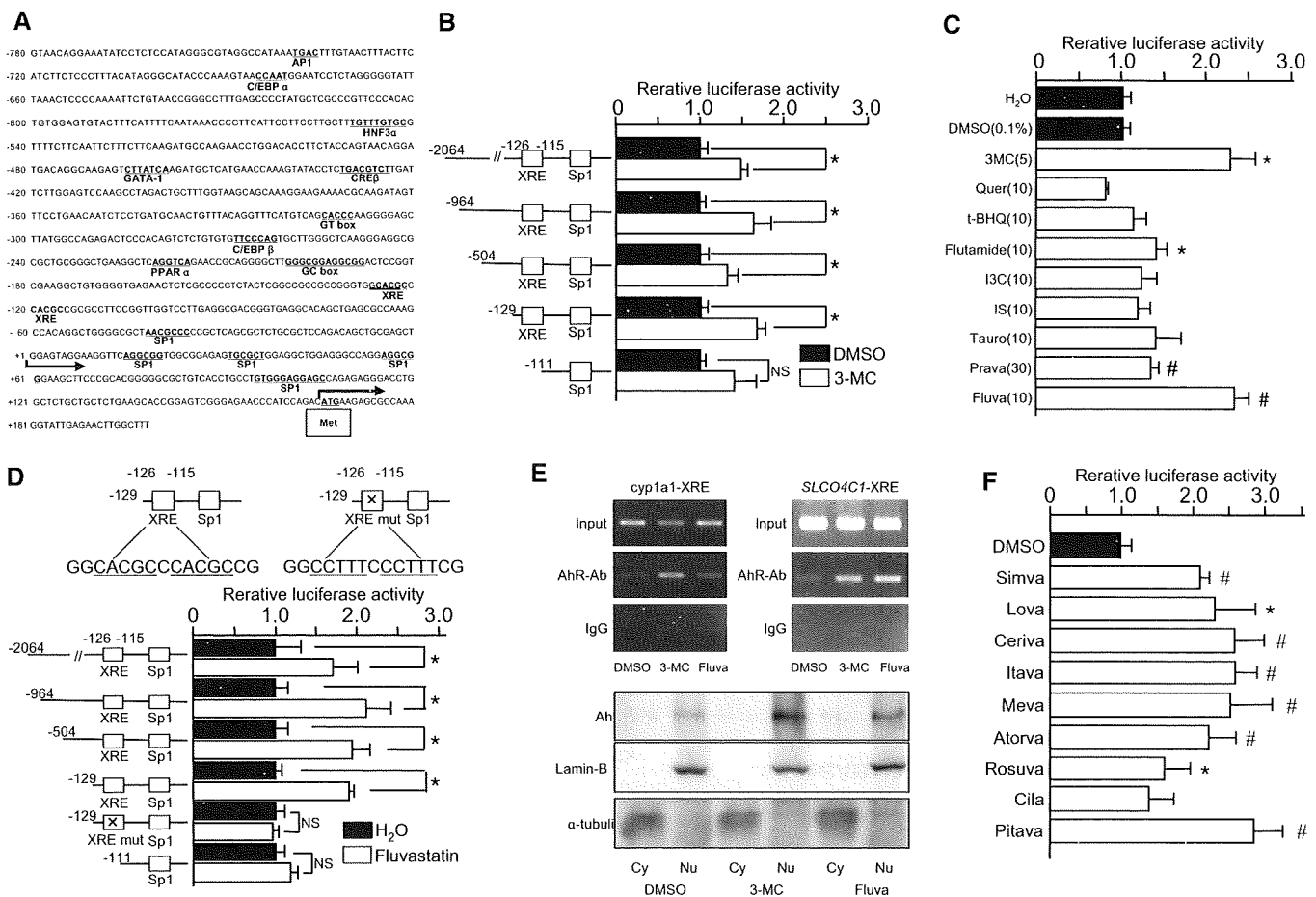


Figure 5. Transcriptional analysis and ligand screening are shown. (A) The 5' region of human SLCO4C1. Potential *cis*-acting sequences are indicated. Met, first methionine. (B) Promoter activity of human SLCO4C1. Deletion constructs of the human SLCO4C1 promoter region were analyzed with 3-MC (5 μ M). * P < 0.05 (n = 3 to 4 per group). (C) Enhancement of promoter activity of human SLCO4C1 with various compounds (concentration as indicated, μ M). Quer, quercetin; t-BHQ, *tert*-butylhydroquinone; I3C, indole-3-carbinole; IS, indoxyl sulfate; Tauro, taurocholic acid; Prava, pravastatin; Fluva, fluvastatin. * P < 0.05 versus DMSO; # P < 0.05 versus H₂O (n = 3 to 4 per group). (D) Effect of fluvastatin (10 μ M) on human SLCO4C1 transcription. Deletion constructs and loss-of-function mutation construct in XRE motifs of human SLCO4C1 were examined. * P < 0.05 (n = 3 to 4 per group). (E) ChIP assay and Western blotting of 3-MC or fluvastatin-treated cells. (Top) After application of 3-MC (1 μ M) or fluvastatin (10 μ M), fixed cell extract was analyzed by mouse cyp1a1 XRE or human SLCO4C1 XRE PCR. (Bottom) Western blotting of nuclear and cytoplasmic fractions from HEK293T cells were stained with antibodies against AhR, Lamin B, or α -tubulin antibodies. Cy, cytosolic fraction; Nu, nuclear fraction. (F) Enhancement of human SLCO4C1 promoter activity with various statins (10 μ M) using the minimal promoter region (-129). * P < 0.05; # P < 0.01 (n = 3 to 4 per group).

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.^{2,3} GSA accumulation causes various harmful effects, such as inhibition of platelet aggregation hemolysis and convulsions.²⁶ Likewise, ADMA is the most specific endogenous compound with inhibitory effects on NO synthesis, and it has also been implicated in the development of hypertension and adverse cardiovascular events.^{6,7} *Trans*-aconitate, known as anti-feedant in brown plant hoppers,²⁷ is an inhibitor of aconitase and 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.²⁸ *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-

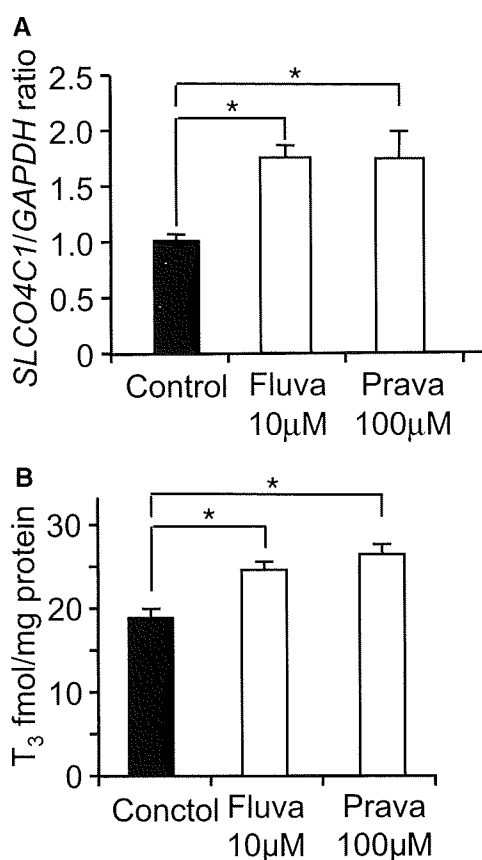


Figure 6. Effects of statins on SLCO4C1 expression and function *in vitro*. (A) Real-time PCR of SLCO4C1 in ACHN cells with fluvastatin (10 μ M) or pravastatin (100 μ M; $n = 3$ per group). (B) The uptake of T_3 by ACHN cells treated with fluvastatin (10 μ M) and pravastatin (100 μ M). * $P < 0.05$ ($n = 3$).

scription to facilitate the excretion of uremic toxins like a transgene phenotype. In patients with CKD, therapy with statins has the potential not only to lower cardiovascular morbidity and mortality but also to slow the progression of renal disease.²² The effects are thought to be dependent on such mechanisms as a reduction of endothelial dysfunction, inhibition of inflammatory responses, and reduction of oxidative stress.^{22,29} Recently, the relationship between statin administration and ADMA was examined in humans. The serum level of ADMA in metabolic syndrome was reduced by fluvastatin.³⁰ Thus, our data provide new scientific bases for renal protection to facilitate the excretion of uremic toxins in patients with CKD by drugs including statins as “transporter potentiators” (Figure 8). Because the significantly increased levels of GSA and ADMA were reported in patients with autosomal dominant polycystic kidney disease (ADPKD),⁵ our data also support the clinical study and will be a new clue for further protection of renal damage in patients with ADPKD.

Cytochrome P-450 (CYP) comprises a superfamily of enzymes that catalyze oxidation of numerous xenobiotic chemicals, including drugs, toxic chemicals, and carcinogens, as well as endobiotic chemicals.³¹ Among these CYP enzymes, *cyp1a1* is important in the metabolism of carcinogens such as dioxin and halogenated

aromatic hydrocarbons.³¹ Because of the prominently catalyzing role, it has been believed that compounds that induce *cyp1a1* activation are detrimental to humans and animals; however, it is also reported that induction of *cyp1a1* is a sensitive but nonspecific indicator of AhR binding and activity, and the induction of *cyp1a1* and activation of AhR are not synonymous with dioxin-like toxicity, including carcinogenesis.³² Clinically, various weak AhR ligands, such as flutamide, omeprazole, and atorvastatin, were identified³² but the Food and Drug Administration approves usage of these compounds, and in fact, they do not produce dioxin-like toxicities, including carcinogenesis in humans. Because statins have been used for a long time with a high safety and tolerability profile, induction of SLCO4C1 by statins in the kidney in patients with CKD and ADPKD may be a safe and new therapeutic tool to excrete uremic toxins and for reduction of renal inflammation.

We also found that the activation potency of the AhR-XRE system differs between *cyp1a1* and *slco4c1* in the kidney. In the rat liver, *cyp1a1* was significantly induced by flutamide (329-fold) and omeprazole (79-fold), although renal *cyp1a1* was weakly up-regulated by flutamide (three-fold) and omeprazole (15-fold; Supplemental Figure 3, A and B). It is also reported that some statins significantly induced *cyp1a1* in kidney but rather weakly in the liver, suggesting that statins act as AhR ligands mainly in the kidney.³² Conversely, the renal activation of *slco4c1* by flutamide and omeprazole was quite weak (Supplemental Figure 3C). Thus, further exploring for drugs that upregulate human SLCO4C1 only in the kidney much more potently than statins should be a new clinical tool for patients with CKD and ADPKD to decelerate renal damage and to delay initiating hemodialysis.

Metabolomics is an emerging tool that can be used to gain insights into cellular and physiologic responses. By CE-MS, we identified various renal failure-related compounds (Supplemental Figure 2, Supplemental Tables 1 through 4). In renal failure, indoxyl sulfate, creatinine, GSA, and guanidinoacetate were reported as uremic toxins.⁴ Increase of citrulline and trimethyl N-oxide,³³ 3-methylhistidine,³⁴ N,N-dimethylglycine,³⁵ and allantoin³⁶ and decrease of carnitine,³⁷ Trp, and Tyr³⁸ were also reported in renal failure.

On the other hand, increase of *trans*-aconitate, 4-acetylbutyrate, hexanoate, argininosuccinate, α -amino adipate, and pipercolate and decrease of desethyltrazine and methionine sulfoxide so far have not been reported in renal failure (Supplemental Figure 2). Thus, our data will be useful for clarifying the metabolic pathway of renal failure.

CONCISE METHODS

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 SLCO4C1¹⁰ was inserted into the pGEM-sgl2-5pr-mut plasmid containing kidney-specific *sglt2* pro-

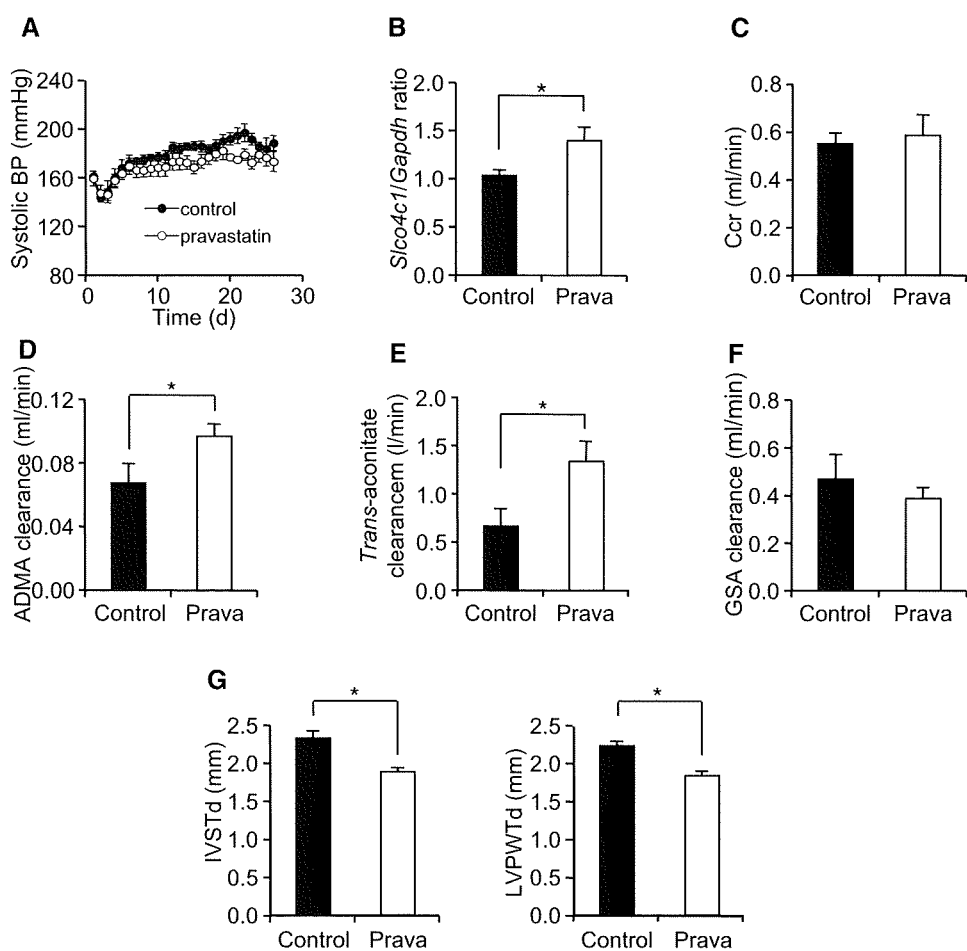


Figure 7. Effects of pravastatin *in vivo*. (A) BP in control and pravastatin-treated (0.1 mg/ml drinking water) rats after five-sixths Nx ($n = 6$ to 7 per group). (B) The mRNA expression of rat *slco4c1* in the kidney after pravastatin administration ($n = 11$ per group). (C through F) Renal clearance 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). * $P < 0.05$.

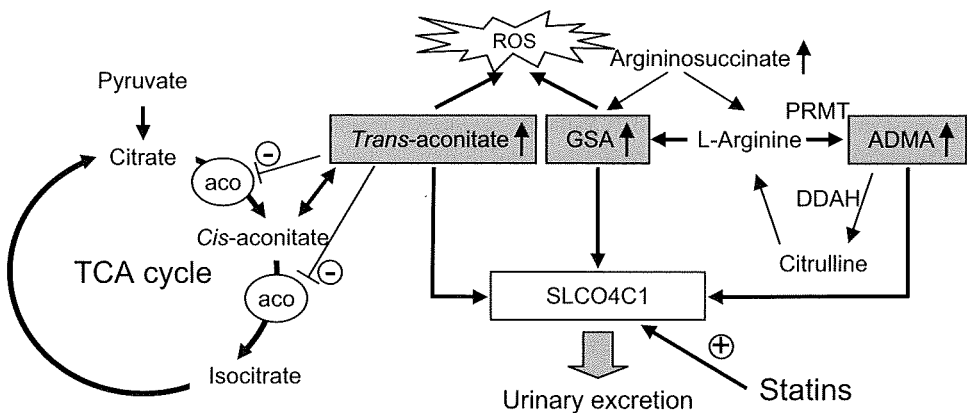
motor.¹¹ The linear purified plasmid was injected into the pronuclei of fertilized oocytes of Wistar rats. Pups were analyzed for the genomic integration by Southern blotting and by PCR amplification of tail DNA using the following primers: Forward (mouse *sglt2*) 5'-tccccactctgtt-tcccagctatgt-3' and reverse (human *SLCO4C1*) 5'-acggcatctgcagaatt-agcttgggctc-3'. Reverse transcriptase-PCR was carried out using the same primers that can amplify the full length of human *SLCO4C1* cDNA. Resultant TG(+) rats showed normal breeding and development with no obvious phenotypic abnormalities in body weight, water and food intake, and renal functions compared with TG(-) littermates, whose genetic background is the same as that of TG(+) 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,³⁹ 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 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*-aconitate 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.¹⁰ 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).⁴⁰

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.⁴¹

CE-MS Method for Metabolome Analysis

A comprehensive and quantitative analysis of charged metabolites by CE-MS was performed.¹³ 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 $eGFR \text{ (ml/min per } 1.73 \text{ m}^2) = 175 \times \text{creatinine}^{-1.154} \times \text{age}^{-0.203} \times 0.742 \text{ (if female)} \times 0.741$.

Measurement of Reactive Oxygen Species

The free radical formation within the human kidney proximal cell line HK-2 evoked by *trans*-aconitine (100 μ M) 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)⁴³ 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 μ g of *Renilla* Luciferase Reporter Vector PhRL-TK (Promega) as well as co-transfection with AhR and AhR nuclear translocator expression vector.¹⁸ Forty-eight hours after ligand treatment, reporter assay was performed using Dual Luciferase Reporter Assay System (Promega). Incubation with activators of constitutive androstane receptor (clotrimazole and TCPOBOP), pregnane X receptor (rifampicin), and peroxisome proliferator-activated receptor α (bezafibrate, fenofibrate, clofibrate, and LTB₄) did not affect the SLCO4C1 transcription (data not shown).

ChIP Assay

ChIP assays were performed as described previously.⁴⁴ Briefly, cells either untreated or exposed to 3-MC (mouse HepaC1C7 cells) or fluvastatin (HEK293T cells) were cross-linked with 1% formaldehyde, and protein-DNA complexes were immunoprecipitated using rabbit polyclonal

antibody against AhR (BIOMOL, Plymouth, PA) or nonspecific anti-rabbit IgG. The recovered DNA was then subjected to PCR using primers that amplify regions containing the XRE elements of the human SLCO4C1 gene (forward primer 5'-AAGGGGAGCTTATGGCCA-GAGACTC-3' and reverse primer 5'-TCGCCTCAAGGACCAACCG-GAAG-3') or mouse *cyp1a1* gene (forward primer 5'-CTATCTCTTA-AACCCACCCCAA-3' and reverse primer 5'-CTAAGTATGGT-GGAGGAAAGGGTG-3'). Nuclear and cytoplasmic fraction extracts were prepared and Western blotting was performed as described previously³⁹ 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 \pm 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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See related editorial, "Harnessing Transporters to Clear Uremic Toxins," on pages 2483–2484.

Supplemental information for this article is available online at <http://www.jasn.org/>.

Letter

Carbonated soft drinks and carbonyl stress burden

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ABSTRACT — Carbonated soft drinks reportedly contain methylglyoxal (MG), which is strongly associated with human carbonyl stress. We sought to evaluate the effects of carbonated drink intake on human carbonyl stress. We measured MG levels in 4 commercial beverage brands, and evaluated the changes in plasma MG in healthy subjects following the intake of carbonated drinks. By 30 min after intake of samples containing high glucose and high MG, the levels of plasma MG, glucose, insulin and uric acid had increased significantly, and then returned to basal levels by 120 min. After intake of the low-calorie carbonated samples containing little MG, there were no increases in plasma MG. Our results suggest that glucose-containing carbonated soft drinks are associated with increases in not only glucose but also carbonyl burden.

Key words: Carbonated soft drink, MG, Carbonyl stress

INTRODUCTION

Carbonated soft drink consumption reportedly leads to cardiometabolic risk factors, such as hypertension, impaired glucose tolerance and chronic kidney disease (Dhingra *et al.*, 2007; Winkelmayer *et al.*, 2005; Saldana *et al.*, 2007). Moreover, previous reports have shown that carbonated drinks contain methylglyoxal (MG), which is a highly reactive carbonyl compound and major precursor of advanced glycation end products (AGEs), and displays toxicity in cells and tissues (Tan *et al.*, 2008; Fukunaga *et al.*, 2004; Okado *et al.*, 1996; Ramasamy *et al.*, 2006). Food and beverages represent exogenous sources of MG (Nemet *et al.*, 2006); however, few reports have evaluated the actual effects of drinking and eating such products on plasma MG levels.

Moreover, carbonyl stress caused by the accumulation of reactive carbonyl compounds is also associated with hypertension, diabetic complications and uremic states, and carbonyl stress plays a pathological role in these diseases (Wang *et al.*, 2008; Beisswenger *et al.*, 2003; Miyata *et al.*, 2001; Nakayama *et al.*, 2008). Therefore, whether the intake of carbonated soft drinks affects the carbonyl

stress burden is of clinical importance.

In this study, we measured MG levels in 4 commercial beverage brands, and evaluated the changes in plasma MG levels and metabolic factors, such as glucose and uric acid (UA), after intake of 2 types of carbonated soft drink (regular and low-calorie).

MATERIALS AND METHODS

Beverage samples

We purchased 4 commercially available types of carbonated soft drink, including cola, lemon-lime soft drink, and 2 brands of diet cola (Table 1). Samples A (a cola) and C (a diet cola) were used for the loading tests.

Subjects

Subjects comprised 6 healthy volunteers (age range, 20 to 48 years) for loading tests with sample A, and 5 volunteers (age range, 22 to 48 years) for sample C. All subjects had normal renal function and no metabolic risk factors. After an 8-hr overnight fast, blood samples were obtained (pre) and subjects then consumed 300 ml of sample A or 500 ml of sample C. Blood samples were drawn after 30,

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Table 1. Concentrations of methylglyoxal in 4 types of carbonated drink

Sample	Category	Carbohydrate (g/100 ml)	MG ^a (μM)
A	Cola	11.3	7.2
B	Lemon-lime soft drink	10.0	5.8
C	Diet-type cola	0.0	0.2
D	Diet-type cola	0.0	0.7

^aMG, methylglyoxal

60 and 120 min. The Ethics Committee of Tohoku University approved this study protocol, and informed consent was obtained from all subjects.

Laboratory analyses

After centrifugation of blood, plasma was aspirated and stored at -80°C until assayed. MG levels were assayed by derivatization with *o*-phenylenediamine (*o*-PD) and electrospray ionization liquid chromatography mass spectrometry (ESI/LC/MS) of the resulting quinoxalines, as reported previously (Nakayama *et al.*, 2008). To obtain more precise data, we modified the analytical conditions of LC/MS. The gradient speed of the mobile phase was slowed (from 6 to 10 min) and the mass/charge ratio (*m/z*) was detected more precisely (from *m/z* 145 to *m/z* 145.07). The resulting plasma MG levels from this new method were lower than our previous data derived from the previous method, but high relativity between the new and old methods was observed for 30 plasma samples: 10 from healthy controls and 20 from patients with renal failure (regression equation: $y = 0.89x - 92$, $R^2 = 0.97$). Plasma insulin was measured by the chemiluminescent enzyme immunoassay method, and glucose and other laboratory data were measured using an automatic analyzer at our clinical laboratory. Chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Statistical analyses

SPSS version 11.0 software (SPSS Japan, Tokyo, Japan) was used to evaluate the changes in plasma before and after soft drink intake by analysis of variance with repeated measures and Dunnett's test. Values of $P < 0.05$ were considered to indicate statistical significance.

RESULTS AND DISCUSSION

The MG concentrations in 4 types of carbonated soft

drink are listed in Table 1. Samples A and B contained high concentrations of carbohydrates and significant levels of MG. Samples C and D were low-calorie drinks and contained very low MG levels.

We examined the changes in plasma MG levels after intake of sample A, which contained high glucose levels and the highest level of MG among the 4 drinks, and after intake of sample C, a diet-type drink. As shown in Fig. 1, plasma MG, glucose and insulin were increased in most subjects at 30 min after intake of sample A, while no remarkable change was observed in the test for sample C, except that the glucose level gradually increased. The increases in plasma MG at 30 min after intake of sample A were statistically significant, indicating that glucose-containing carbonated soft drinks may, at least partly, increase the carbonyl burden. In some subjects (4 and 5), the increases in both plasma MG and glucose were observed at 30 min (Figs. 1a and c), and these results may suggest that the increases in MG was due to secondary production from absorbed glucose. However, in other subjects (2 and 3), plasma MG was higher at 30 min without a concomitant increase in plasma glucose. Moreover, the amount of MG contained in sample A was $2.2 \mu\text{mol}$ ($7.2 \mu\text{mol/l}$, 300 ml), which was sufficient to raise the concentration from 110 to 170 nmol/l in 36 l water, which is similar to the body fluid volume in a person weighing 60 kg. Therefore, the increase in plasma MG was most likely due to direct absorption from the drinks. A previous report showed that significantly high levels of MG (from 3.3 to 19.3 μM) were present in 11 brands of carbonated soft drinks (Tan *et al.*, 2008), and their findings are coincident with the results of the present study.

In addition, UA levels were slightly but significantly, higher at 30 and 60 min. The extent of these changes was very small, and thus, its effect may be of little clinical significance. However, a high level of UA is reportedly one of the independent risk factors for cardiovascular disease (Choi and Curhan, 2007), therefore it is of interest whether plasma UA is associated with habitual intake of carbonated soft drinks.

Curiously, in all subjects, although the changes were small, plasma glucose levels gradually increased after intake of sample C, which contained no carbohydrate, while insulin levels did not increase. The threshold level of insulin secretion is thought to be above 100 mg/dl, and thus these increases in glucose were too small to stimulate beta cells to release insulin. These slow changes in glucose may be physiologic phenomena caused by circadian changes in hormones (e.g., insulin, glucagon and cortisol), but the exact mechanisms at work remains unclear.

In conclusion, glucose-containing carbonated soft

Carbonated soft drinks and carbonyl stress

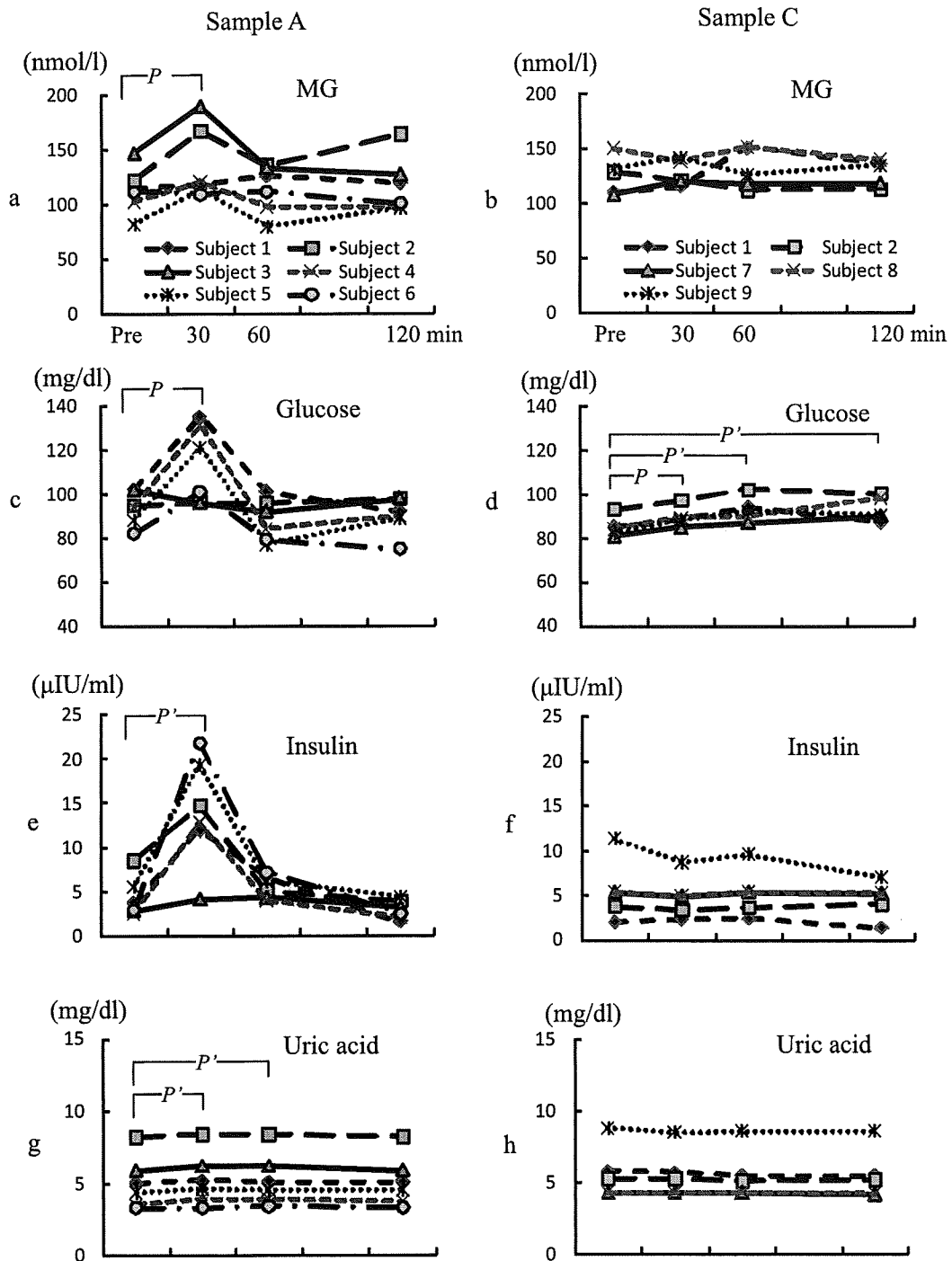


Fig. 1. Changes in plasma MG, glucose, insulin and UA levels before and after intake of sample A (glucose containing carbonated drink) (a, c, e, g) or sample C (diet-type carbonated drink) (b, d, f, h). Subjects 1 and 2 participated in both tests. For sample A, the MG level (mean \pm S.D.) at pre and 30 min was 113 ± 22 and 136 ± 34 nM, respectively (a). The glucose level at pre and 30 min was 94 ± 8 and 113 ± 18 mg/dl, respectively (c). The insulin level at pre and 30 min was 4.4 ± 2.3 and 14.1 ± 6.2 μ IU/ml, respectively (e). The UA level at pre, 30 and 60 min was 5.0 ± 1.8 , 5.3 ± 1.8 and 5.3 ± 1.8 mg/dl, respectively (g). For sample C, the glucose level at pre, 30, 60 and 120 min was 85 ± 5 , 89 ± 5 , 93 ± 6 and 93 ± 6 mg/dl, respectively (d). $P < 0.05$ vs pre, $P' < 0.01$ vs pre.

drinks appear to lead to a transient increase in plasma MG levels. It is of great interest whether habitual intake of carbonated drinks enhances human carbonyl stress and UA levels, or is involved with enhanced cardiovascular events among these subjects. Further studies are required to address these issues.

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Methylglyoxal contributes to the development of insulin resistance and salt sensitivity in Sprague–Dawley rats

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Objectives Methylglyoxal, a metabolite of the glycolysis pathway, may play an important role in the development of diabetes and hypertension, but the exact mechanism has not been fully elucidated. The present study was designed to investigate whether methylglyoxal could directly induce insulin resistance and salt sensitivity in Sprague–Dawley rats.

Methods Rats were allocated to four groups: control (normal drinking water), 1% methylglyoxal in drinking water, 1% methylglyoxal plus *N*-acetyl cysteine (NAC) (800 mg/kg per day), a methylglyoxal scavenger, or TM2002 (100 mg/kg per day), an advanced glycation endproducts (AGEs) inhibitor. After 4-week treatment insulin resistance was evaluated by an euglycemic hyperinsulinemic glucose clamp technique. In another set of rats, either a high-salt diet (4%) alone, standard rat chow with 1% methylglyoxal in drinking water or high-salt diet plus methylglyoxal was given for 4 weeks. Immunohistochemistry was performed to measure nitrotyrosine and methylglyoxal-induced AGEs, N^ε-carboxyethyl-lysine (CEL) in the kidney.

Results Four-week treatment with NAC or TM2002 completely improved methylglyoxal-induced insulin resistance. Co-administration of methylglyoxal and high-salt diet significantly increased systolic blood pressure, urinary albumin excretion, urinary thiobarbituric acid-reactive substances excretion and the renal nitrotyrosine expression in the kidney (markers of oxidative stress)

Introduction

Methylglyoxal (MGO) has been suggested to be involved in the pathophysiology of hypertension [1–3], but the exact mechanism is not yet fully understood. MGO can react with selective proteins to yield irreversible advanced glycation endproducts (AGEs), leading to cross-linking and denaturation of protein [4]. The irreversible reaction of MGO with lysine residues of protein forms N^ε-carboxyethyl-lysine (CEL) and N^ε-carboxymethyl-lysine (CML) [5]. MGO and related AGEs such as CEL are precursors of increased oxidative stress *in vivo* [5,6]. Oxidative stress is determined with increased production or decreased degeneration of reactive oxygen species (ROS), or both, which would associate with MGO and its metabolites. In fact, increased levels of ROS, together with elevated MGO levels, have been reported in both

compared with methylglyoxal or high-salt diet alone. Renal CEL was significantly increased in methylglyoxal-treated rats compared with nonmethylglyoxal-treated rats.

Conclusion These results indicate that methylglyoxal-induced insulin resistance and salt sensitivity at least in part by increasing oxidative stress and/or AGEs formation in Sprague–Dawley rats. The present study provides further evidence for methylglyoxal as one of the causative factors in the pathogenesis of insulin resistance and salt-sensitive hypertension. *J Hypertens* 27:1664–1671 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: advanced glycation endproducts, diabetes, hypertension, insulin resistance, methylglyoxal, oxidative stress, salt sensitivity

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cultured vascular smooth muscle cells and vascular tissues from hypertensive animals [3,7,8]. In addition, positive CEL and CML staining has been enhanced in aortic tissues even from 5-week-old spontaneously hypertensive rats (SHR), but not in age-matched Wistar–Kyoto (WKY) rats [3]. The results of these studies indicate that elevated MGO levels can lead to increased production of ROS and AGEs, and therefore might contribute to the development of hypertension.

On the contrary, increased accumulation of MGO and/or its metabolites has been observed in various insulin resistance states, such as in diabetic patients [9] and hypertensive animals [2,3]. Furthermore, epidemiological studies have shown that about 50% of salt-sensitive individuals are insulin resistant [10]. These findings

suggest that the accumulation of MGO may play an important role in the development of insulin resistance and salt-sensitive hypertension.

Thus, we hypothesized that MGO could directly induce insulin resistance and salt sensitivity. To test this hypothesis, the present study was designed to determine whether 4-week treatment with MGO would induce insulin resistance and salt sensitivity in normotensive Sprague–Dawley rats. The role of MGO in the salt sensitivity was determined in Sprague–Dawley rats because treatment with a 4% high-salt diet for 4-weeks did not significantly increase blood pressure in this strain [11].

Methods

Animals

Eight-week-old male Sprague–Dawley rats (SLC, Shi-zuoka, Japan) were housed in a controlled temperature (24°C) room with a 12-h light–dark cycle. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and protocols were approved by the animal committee of Tohoku University.

Protocol 1: role of methylglyoxal in insulin sensitivity

Rats were divided into four groups: control group (tap drinking water, CON-GC, $n=6$), MGO (1% MGO in tap drinking water) treated group (MGO-GC, $n=7$), 1% MGO plus *N*-acetyl cysteine (NAC) treated group (MGO + NAC, $n=6$), and 1% MGO plus TM2002 treated group (MGO + TM2002, $n=7$). NAC dissolved in water was given twice daily by oral gavage at a dose of 400 mg/kg in MGO + NAC group. TM2002, a potent inhibitor of AGEs, was synthesized by Izuhara *et al.* [12] was dissolved in water and given twice daily by oral gavage at a dose of 50 mg/kg in MGO + TM2002 group. Rats were fed with a standard rat chow (0.5% NaCl MR stock; Nihon Nosan, Yokohama, Japan). Rats were pair-fed to ensure equivalent caloric intake, thereby avoiding the influence of different food intakes on the metabolic abnormalities. Body weight was measured weekly.

Euglycemic hyperinsulinemic glucose clamp technique

At the age of 13 weeks, rats were anesthetized with ether, and an arterial catheter (PE 100) and a venous catheter (PE 20), both filled with heparinized physiological saline (100 IU/ml), were implanted into the left carotid artery and right jugular vein for collecting blood samples and for administration of the infusate, respectively. After overnight fasting, conscious rats were examined for insulin sensitivity by a euglycemic hyperinsulinemic glucose clamp technique. Before the start of the glucose clamp, the fasting blood glucose was measured by a blood glucose test meter (A790820; GUNZE Co., Kyoto, Japan). The initial load of insulin (25 mU/kg of Humalin

R, U-100; Eli Lilly Japan Co., Kobe, Japan) was infused through a venous catheter as a bolus, and this was followed by a constant infusion of insulin at a rate of 4 mU/kg per min for 147 min. During the glucose clamp, 12.5% glucose solution was infused as needed to maintain the blood glucose at the fasting level. Ten microliters of arterial blood were sampled through an arterial catheter at 7-min intervals for determination of the blood glucose. The average of the rate of glucose infusion (mg/kg per min) for the last 35 min was taken as an index of insulin sensitivity (M value) [13].

Protocol 2: role of methylglyoxal in salt sensitivity

Rats were randomly allocated to three groups and fed a high-salt diet (4% NaCl; high-salt diet, $n=6$) and tap water, standard rat chow with 1% MGO in tap drinking water (MGO, $n=6$) or high-salt diet plus MGO (high-salt diet + MGO, $n=6$) for 4 weeks. We also pair-fed rats to ensure equivalent caloric intake as in protocol 1. Body weight was measured weekly. Systolic blood pressure (SBP) was measured every week in conscious rats by an indirect tail-cuff method (Model MK-2000A; Muromachi, Tokyo, Japan). Twenty-four-hour urinary samples were collected under an ice-cooled condition in a metabolic cage at weeks 0 and 4. Urinary sodium Na^+ , albumin and creatinine were measured by a standard autoanalysis technique (Synchron-CX-3; Beckman Coulter Inc., Fullerton, California, USA).

Thiobarbituric acid-reactive substances

We determined the degree of lipid peroxidation using biochemical assays of thiobarbituric acid-reactive substances (TBARS) in urine, as described previously [14]. Briefly, using tetraethoxypropane as a standard, 0.2 ml of urine or standard samples were incubated with 0.4 ml of (1:1:1 ratio) TBA–HCl–TCA reagent (thiobarbituric acid 0.325%, 0.25 N HCl and 15% thi-chloroacetic acid) for 15 min in boiling water, then cooled and centrifuged at room temperature for 10 min at 3000 *g*. The absorbance of clear supernatant was measured against a reference blank at 535 nm.

Immunohistochemistry of the kidney

At the end of the experiment, the rats were sacrificed by intraperitoneal injection of pentobarbital (100 mg/kg). For immunohistochemical analysis, the left kidneys were fixed for 48 h – that is, for 24 h in 10% formalin and for 24 h in 100% ethanol. They were then embedded in paraffin and sectioned at a thickness of 2- μm . Sections were immunostained with CEL as a marker of AGEs and carbonyl stress, and with nitrotyrosine as a marker of peroxynitrite formation [15]. For CEL and nitrotyrosine immunostaining, deparaffinized sections were retrieved by microwaving in 10 mmol/l citrate buffer (pH 7.0) for 5 min, staining with anti-CEL (KH-025, diluted 1:50; Trans Genic, Kumamoto, Japan) or antinitrotyrosine (AB5411, diluted 1:200; Chemicon, Temecula,

California, USA), and visualized with diaminobenzidine containing nickel chloride. The percentage of the cortex and outer medullary region positive for CEL or nitrotyrosine was determined in at least 30 randomly chosen frames at 20× magnification captured by an optical microscope connected to a CCD video camera (BX51, DP70; Olympus Optical Co., Tokyo, Japan), and the data were analyzed with the Image J software.

Data analysis

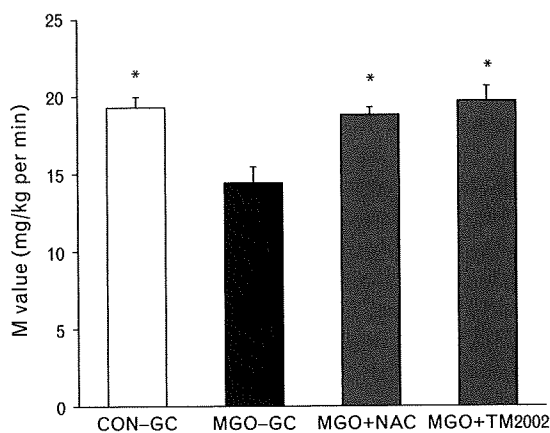
All results were expressed as the means ± SE. Intergroup comparisons of SBP over the study period were made by repeated measures ANOVA with Bonferroni/Dunn test. All other data analyses were performed by Bonferroni/Dunn test for multiple comparisons after one-way analysis of variance. Values of *P* < 0.05 were considered to indicate statistical significance.

Results

Insulin sensitivity

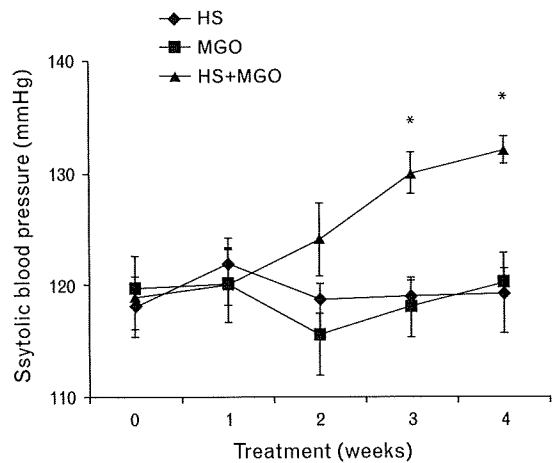
After 4 weeks of treatment, there were no significant intergroup differences in body weight (BW; CON-GC, 304 ± 5 g; MGO-GC, 312 ± 7 g; MGO + NAC, 308 ± 10 g; MGO + TM2002, 307 ± 6 g) or in fasting blood glucose (CON-GC, 6.4 ± 0.2 mmol/l; MGO-GC, 6.4 ± 0.3 mmol/l; MGO + NAC, 6.4 ± 0.2 mmol/l; MGO + TM2002, 6.5 ± 0.3 mmol/l). The M value in MGO-GC (14.4 ± 1.1 mg/kg per min) was significantly lower than that in CON-GC (19.3 ± 0.7 mg/kg per min), MGO + NAC (18.8 ± 0.5 mg/kg per min), and MGO + TM2002 (19.7 ± 1.0 mg/kg per min), respectively, as shown in Fig. 1. However, there were no significant differences in the M value among CON-GC, MGO + NAC and MGO + TM2002 three groups.

Fig. 1



Comparison of the index of insulin sensitivity (M value) in the four groups. CON-GC (*n* = 6), control; MGO-GC (*n* = 7), treatment with methylglyoxal; MGO + NAC (*n* = 6), co-treatment with methylglyoxal and *N*-acetyl cysteine; MGO + TM2002 (*n* = 7), co-treatment with methylglyoxal and TM2002. Data are the means ± SE. **P* < 0.01 vs. MGO-GC group.

Fig. 2

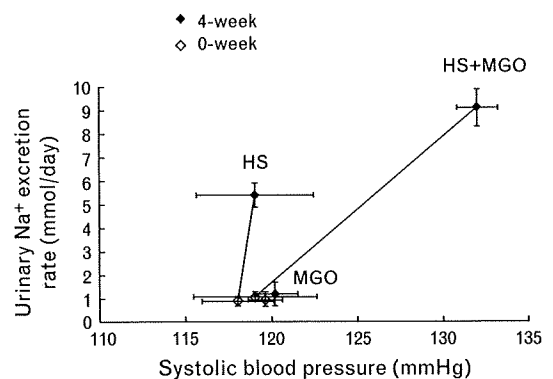


Systolic blood pressure responses to high salt diet or/and methylglyoxal (MGO) treatment at baseline and throughout the 4-week study. Data are the means ± SE. *n* = 6 for each group. **P* < 0.01 vs. HS or MGO group. HS, high-salt diet; MGO, methylglyoxal.

Blood pressure

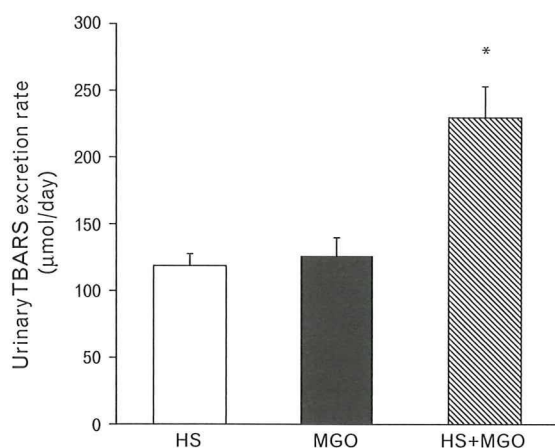
The SBPs of the rats during the 4-week experimental period are shown in Fig. 2. At week 0, there were no significant differences in SBP among the groups. Neither MGO alone nor high-salt diet alone significantly changed SBP during the 4-week experimental period (MGO, 120 ± 1 mmHg to 120 ± 2 mmHg; high-salt diet, 118 ± 2 mmHg to 119 ± 4 mmHg). However, the combination of high-salt diet and MGO progressively increased SBP throughout the experimental period (119 ± 4 mmHg to 132 ± 1 mmHg). High-salt diet + MGO induced a significant increase in SBP compared with the MGO or high-salt diet group (repeated measures ANOVA, *P* < 0.001 or *P* < 0.01, respectively).

Fig. 3



Correlation between the urinary Na⁺ excretion rate and SBP in the three groups at weeks 0 and 4. Data are the means ± SE. *n* = 6 for each group.

Fig. 4



Comparisons of the urinary thiobarbituric acid-reactive substances (TBARS) excretion rate in the three groups after the 4-week study. HS, high-salt-fed rats; MGO, normal-fed rats treated with methylglyoxal; HS + MGO, high-salt-fed rats treated with methylglyoxal. Data are the means \pm SE. $n=6$ for each group. * $P < 0.001$ vs. HS or MGO group.

Twenty-four-hour urinary excretion of Na^+

Figure 3 shows the relationship between urinary Na^+ excretion rate and SBP. When rats were treated with 0.4% salt diet, SBP and urinary Na^+ excretion rate was not different among groups. Although rats treated with 4% salt alone increased urinary Na^+ excretion rate without increase in SBP, rats treated with 4% salt diet together with MGO increased both Na^+ excretion rate and blood pressure indicating that MGO increased salt sensitivity.

Twenty-four-hour urinary excretion of thiobarbituric acid-reactive substances

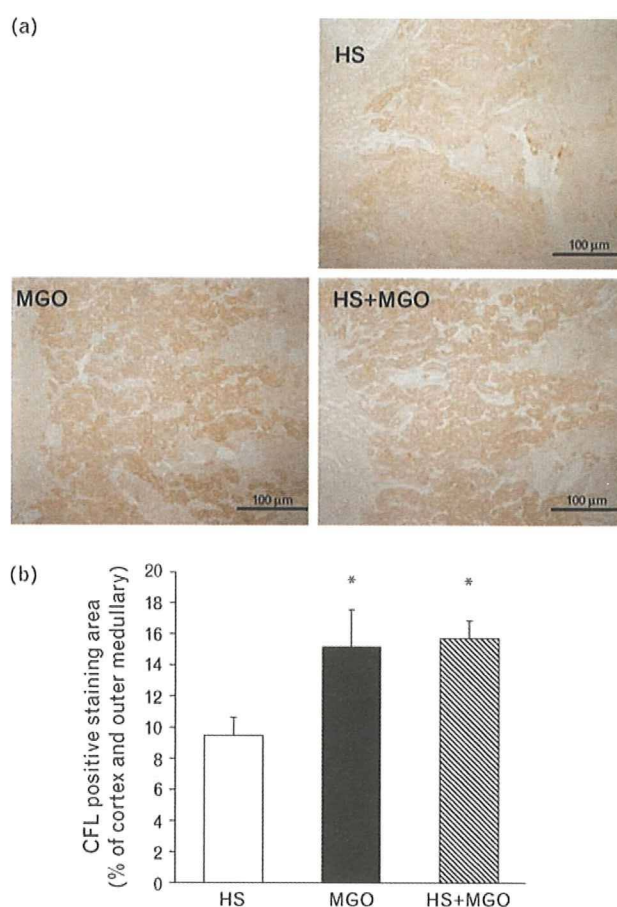
The 24-h urinary excretion of TBARS value in the high-salt diet + MGO group ($229 \pm 23 \mu\text{mol/day}$) was obviously higher than that in either the MGO ($125 \pm 14 \mu\text{mol/day}$) or high-salt diet ($118 \pm 9 \mu\text{mol/day}$) group (Fig. 4).

Immunohistochemistry of the kidney

As shown in Fig. 5, the CEL-positive regions were observed mostly in the glomerular mesangial area and renal tubules. Treatment with MGO alone ($15.2 \pm 2.3\%$) or a combination of high-salt diet and MGO ($15.7 \pm 1.0\%$) for 4 weeks led to a significant increase in the percentage of the cortex and outer medulla stained positive for CEL compared with the high-salt diet alone ($9.5 \pm 1.1\%$). However, there were no significant differences in the percentage of these regions stained positive CEL between the MGO and high-salt diet + MGO groups.

The percentage of the cortex and outer medulla stained positive for nitrotyrosine in the high-salt diet + MGO ($11.5 \pm 0.8\%$, $P < 0.001$) group was clearly higher than that in the high-salt diet ($7.2 \pm 0.8\%$) or MGO ($6.7 \pm 0.4\%$) group (Fig. 6).

Fig. 5



Representation of N^ε-carboxyethyl-lysine (CEL) positive staining area in rats. (a) Representative images of immunohistochemical detection of CEL in the kidney from Sprague–Dawley rats. (b) Percentage of CEL positive staining area of the cortex and outer medulla in HS, MGO and HS + MGO. HS, high-salt-fed rats; MGO, normal-fed rats treated with methylglyoxal; HS + MGO, high-salt-fed rats treated with methylglyoxal. Data are the means \pm SE. $n=6$ for each group. * $P < 0.05$ vs. HS group.

Body and urine compositions

There were no significant intergroup differences in BW, urinary albumin excretion (Ualb) and Ualb/creatinine ratio (Table 1). The kidney weight (KW) was significantly greater in the high-salt diet + MGO group than in the high-salt diet or MGO group. The KW/BW ratio was significantly higher in the high-salt diet + MGO group than in the high-salt diet group. Although it did not reach the level of significance ($P=0.13$), the KW/BW ratio tended to be higher in the high-salt diet + MGO group than in the MGO group.

Discussion

Methylglyoxal increases insulin resistance in Sprague–Dawley rats

That MGO is increased in such insulin resistance states as diabetes and hypertension [2,3,9] indicates that this