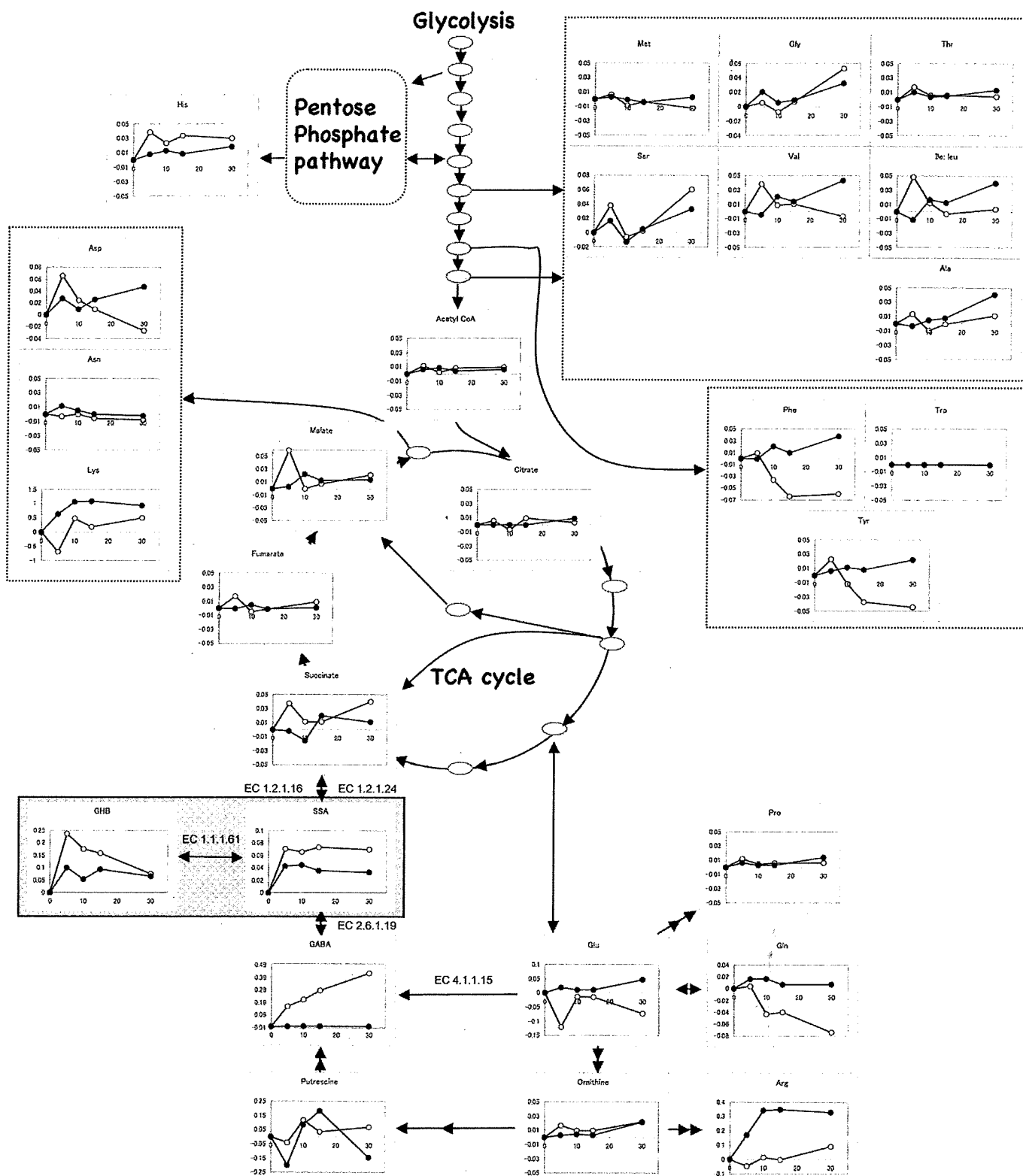


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**FIGURE 6. Time course of intracellular metabolite levels in *E. coli* subjected to SSA treatment.** Measurement of metabolites was performed by CE-TOFMS. The x axis and y axis indicate time (min) after SSA addition and metabolite level (nmol/OD/ml), respectively. The level of metabolite is displayed as amount of metabolite (nmol) in 1 ml of culture ( $OD_{590} = 1$ ) and represents the difference between untreated and treated samples. The *open* and *closed circles* represent the control and YihU overproducing strain, respectively. The expected YihU reaction (EC 1.1.1.61) is highlighted by a *shadowed box*. The data shown are from a representative dataset from three independent experiments. The other two datasets are in supplemental Fig. S5.

contribute to the acid stress response and may also be partially responsible for GABA accumulation (37). This phenomenon did not seem to occur to a significant extent in the

YihU expressing strain probably due to faster conversion of SSA to GHB. In contrast, the levels of some amino acids decreased following SSA treatment in the control strain

(Gln, Phe, and Tyr). These may represent secondary or indirect effects of SSA accumulation as suggested by the delayed responses (>5 min) in contrast to the more rapid changes in metabolites adjacent to SSA (GABA and GHB). Other metabolites such as His and Asp, also showed significant differences in the *t* test (supplemental Table S1), however, the mechanistic link between these responses and SSA treatment is not readily apparent.

Overall, the observed responses in metabolites following SSA treatment support the existence of an alternative SSA metabolic route leading to GHB formation via YihU in *E. coli*. Furthermore, the observed responses to YihU overproduction in *E. coli* cells together with the *in vitro* enzymatic activity results strongly suggest that YihU is a physiologically active SSA reductase.

## DISCUSSION

In this study, we reported a novel biochemical and physiological function for the uncharacterized *E. coli* protein YihU. Screening using the MERMAID method suggested that YihU displays an oxidoreductase activity that was further characterized and found to correspond to that of the GHBDH. Our results further suggest that YihU can play an important role in preventing cellular damage resulting from SSA accumulation by reducing it to GHB.

Our screening method takes advantage of the CE-TOFMS ability to profile a large number of charged metabolites. In this study we did not extend our analysis to lipids or neutral compounds using alternative analytical methods such as LC-MS or GC-MS although it could be advantageous to do so to target a broader range of enzymes. However, neither the current experimental results, nor the sequence similarity of YihU with other enzymes suggest that high affinity substrates are likely to be found among non-polar metabolites. We also used a quick filtration and methanol-quenching method prior to intracellular metabolite extraction as previously optimized for CE-MS analysis (21). Although this method can reproducibly extract most metabolites in the central metabolic pathways, the level of some intermediates may be affected due to rapid intracellular turnover or leakage during the filtration/washing steps that are necessary to ensure reliable measurements by CE-MS. Recently, sampling procedures for microbial cell metabolome analysis have been developed and discussed (38–40). Quenching of the metabolism before filtration and analysis of both intracellular metabolites and culture media may offer promising solutions to some of these issues, but rigorous quantitative comparison of metabolites under different conditions (*i.e.* salt concentration), remains difficult. Despite efforts by many groups there is, to our knowledge, no processing/quenching method that does not result in some significant trade off. Improved solutions are important and challenging tasks for future developments in metabolomics. However, our conclusions, which are based on comparisons of metabolite levels between different treatments and strains, should not be affected by sample processing-related changes in absolute concentration.

Structural studies have shown that a serine residue at the active site of 6-phosphogluconate dehydrogenase is essential

for 6-phosphogluconate binding (7). Interestingly, whereas this serine residue is conserved in all three YihU paralogs of *E. coli* and also in *Arabidopsis* GHBDH, a glycine residue is found at the same position in YihU. In contrast, in the highly conserved catalytic domain consensus sequence GXXGXGXXXKXXXN/Q, the lysine residue previously proposed as a key catalytic residue (7) is conserved in all aligned proteins including YihU. These findings suggest that YihU possesses a  $\beta$ -hydroxyacid dehydrogenase-like catalytic function, whereas its substrate specificity may differ due to the specific amino acid differences found in the substrate-binding domain. In fact, *in vitro* enzymatic activity assays have demonstrated that the YihU protein exhibits substrate specificity and characteristics that are distinct from its paralogs, GlxR and GarR, in agreement with the differences in conserved amino acid residues at the predicted substrate-binding site.

YihU displayed NADH-dependent SSA reductase activity, converting SSA to GHB. SSA is a key intermediate in the GABA metabolic pathway. Although the exact physiological function of this pathway is rather unclear in many organisms, a recent study demonstrated that SSA is a key signal that couples the stress response to quorum-sensing signal decay in bacteria (32). In *Arabidopsis*, GHB accumulates during oxygen deficiency and plays a role in oxidative stress tolerance (14). In animals, GHB is an important neuromodulator in the central nervous system (41). Although the exact physiological role of GHB in *E. coli* remains unclear, YihU may function under specific stress conditions that lead to accumulation of SSA. These may include the acid stress response, a phenomenon associated with the stationary phase of growth. Interestingly a well known mechanism employed to respond to acidification is via glutamate decarboxylase activity, which results in the capture of protons and the formation of GABA (37). It is therefore tempting to speculate that the observed changes in GABA levels are linked to this process. The levels of other metabolites may also have changed in a biologically meaningful way in response to SSA but the current results, using a limited number of replicates ( $n = 3$ ), preclude further conclusions. However, the few statistically significant changes observed support the role of YihU in SSA metabolism and detoxification. SSA is also known to be oxidatively metabolized to succinate by SSA dehydrogenases encoded by *gabD* (10) and *yneI* genes (33) in *E. coli*. However, because YihU activity reduces SSA to GHB, whereas both *gabD* and *yneI* encode SSA dehydrogenases that oxidize it to succinate, it is likely that YihU functions under redox conditions that preclude oxidative SSA metabolism. This hypothesis is supported by the fact that in some plants SSA dehydrogenase activity appears dependent on the energy and NAD<sup>+</sup>/NADH redox state of the cell (42).

An operon that includes the *yihU* gene in *Salmonella* was previously suggested to be important for the formation of surface antigens and resistance to desiccation but its exact biochemical function has not been elucidated (43). The order and structure of genes around *yihU* are well conserved in enterobacteria (*E. coli*, *Salmonella*, and *Shigella*), and, at least in *E. coli*, *yihU* may be part of a transcription unit that includes the adjacent genes *yihS* and *yihT* (EcoCyc (44)).

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Moreover, *yihU* gene expression in *E. coli* appears to be *rpoS*-dependent according to the *E. coli* Gene Expression Data base of the University of Oklahoma. These findings suggest that YihU functions mainly at the stationary phase of growth when the cell faces specific physiological stresses linked to nutrient deprivation.

Methylglyoxal is another aldehyde that can be a substrate for YihU, albeit with lower affinity. Methylglyoxal is a highly toxic electrophile and is known to be produced by microorganisms including *E. coli*. Although the mechanism of its accumulation under various physiological conditions is still relatively unclear, the link between nutritional balance and methylglyoxal production in *E. coli* has been studied (45). These studies suggest that methylglyoxal accumulation may occur under nutritional stress. Similarly to what we found with SSA, *E. coli* cells overproducing YihU appear resistant to higher concentrations of methylglyoxal than wild type.<sup>3</sup> Both GSH-dependent (46, 47) and -independent (48–51) pathways for methylglyoxal detoxification are known in *E. coli*. Although the enzymes involved in the GSH-dependent process are well characterized, some of those associated with the GSH-independent pathways are not well characterized and it is possible that YihU might also play a role in this pathway.

Finally, in anaerobic bacteria such as *Clostridium* sp., GABA is metabolized to butyrate via SSA, GHB, and various CoA-esters (52). The identification of other *E. coli* enzymes catalyzing such additional metabolic reactions downstream of GHB would clarify whether such a pathway is also active in *E. coli*. In conclusion, the main function of YihU in *E. coli* appears to be related to the catabolism of SSA and possibly other toxic aldehydes such as methylglyoxal produced following particular nutritional and energy/redox stresses.

*Acknowledgments*—We are grateful to Hirotada Mori and Kenji Nakahigashi for providing the *E. coli* strains of ASKA clones and Keio collection and Masahiro Sugimoto and Kosaku Shinoda for assistance in metabolome data analyses.

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<sup>3</sup> N. Saito and M. Robert, unpublished data.

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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 m<sup>2</sup> are defined as having chronic kidney disease (CKD).<sup>1</sup> 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.<sup>2,3</sup> So far, more than 110 organic compounds have been identified as uremic toxins.<sup>4</sup> Among these, guanidino compounds, including guanidino succinate (GSA) and asymmetric dimethylarginine (ADMA), are increased in patients with CKD and correlate with prognosis.<sup>3,5</sup> In particular, ADMA, an inhibitor of nitric oxide synthase, is implicated in hypertension, renal damage, cardiac hypertrophy, and cardiovascular events.<sup>6,7</sup> Currently, administration of the oral adsorbent AST-120 is the only therapy to remove uremic toxins in patients with CKD and diabetic nephropathy.<sup>8</sup> Although AST-120 removes indoxyl sulfate, other compounds are not eliminated.<sup>9</sup> 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.<sup>10</sup> 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.<sup>10</sup> Among these, in the kidney, SLCO4C1 might be a first step of transport pathway of digoxin and various compounds into urine.<sup>10</sup> 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.<sup>10</sup> 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.<sup>10</sup> 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.<sup>10</sup> 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 patho-physiologic roles of human SLCO4C1.

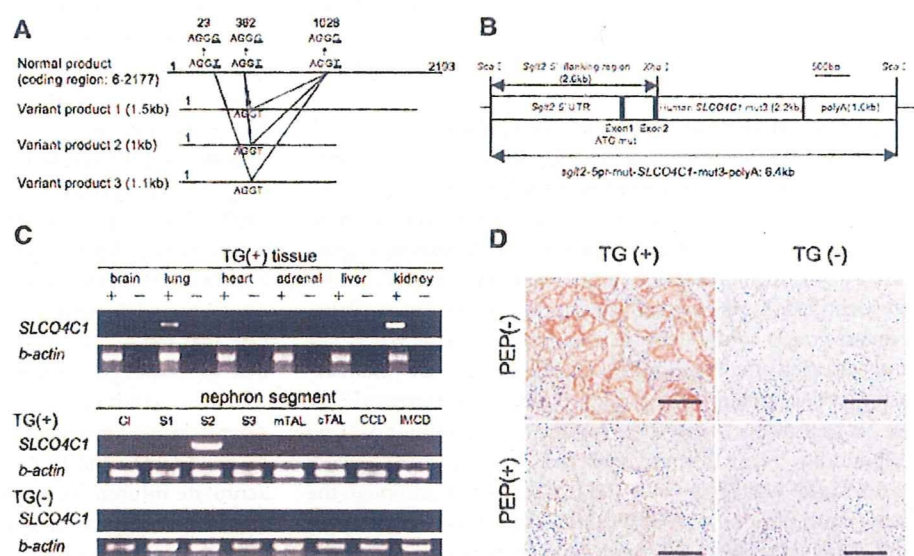
## RESULTS

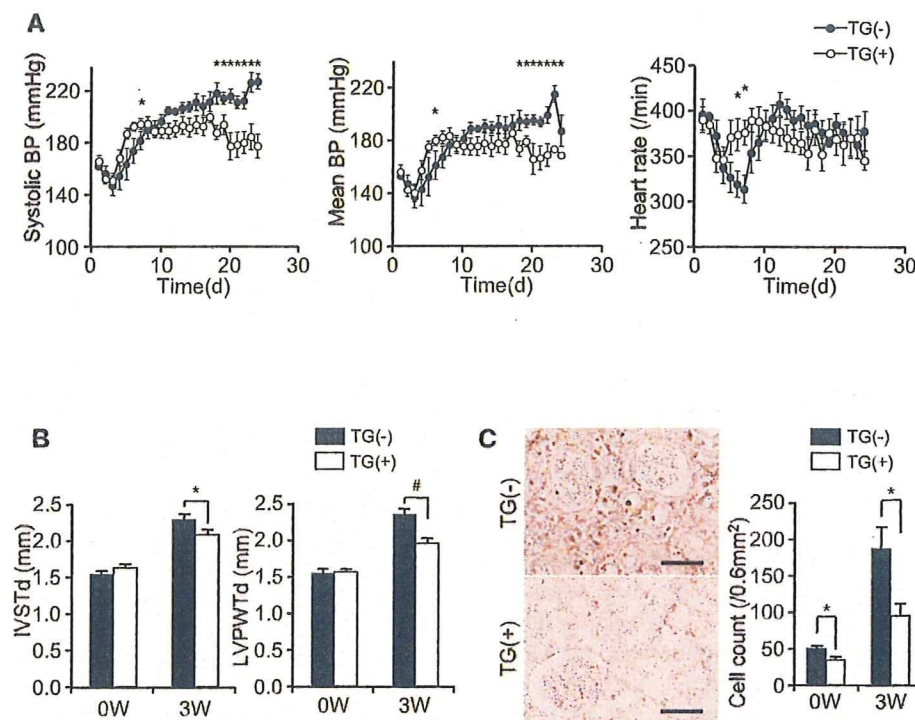
### Generation of TG Rat Harboring Human SLCO4C1 in the Kidney

TG rat harboring human SLCO4C1 in the kidney was generated using the proximal tubule-specific promoter<sup>11</sup> (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  $\mu$ 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<sup>+</sup> 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  $\mu\text{m}$ .

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.<sup>12</sup> 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.

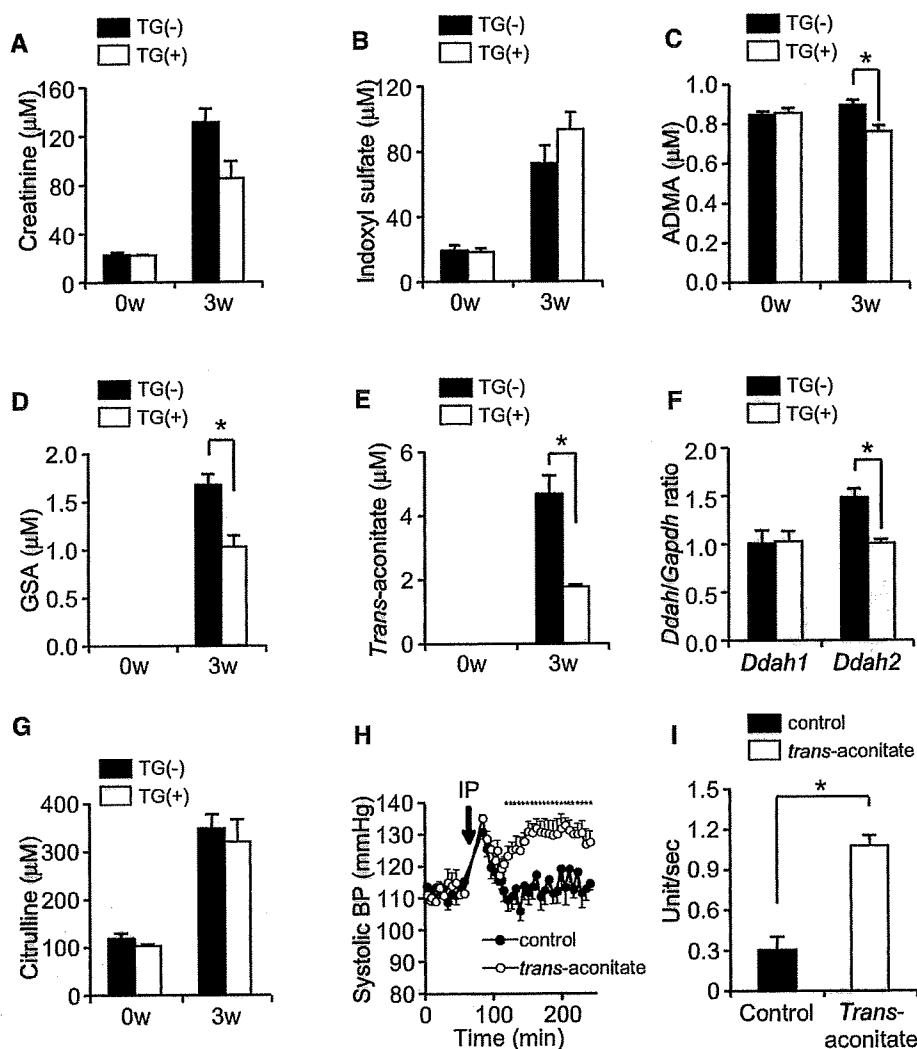
#### Elimination of Uremic Toxins in TG(+ ) Rats

To understand the mechanism by which SLCO4C1 exerted anti-hypertensive and anti-inflammation effects, we performed comprehensive quantitative metabolome analysis.<sup>13</sup> 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,<sup>4</sup> 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.<sup>14</sup> 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,<sup>15</sup> 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.<sup>16</sup> 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.<sup>16</sup> The retention compounds that are biologically/biochemically active and responsive for the uremic syndrome are called uremic toxins.<sup>4</sup> 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 CKD<sup>17</sup>; 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 $\alpha$ , CCAAT/enhancer-binding protein (C/EBP) $\alpha$ , C/EBP $\beta$ , cAMP response element-binding protein (CREB), and peroxisome proliferator-activated receptor  $\alpha$  were found. We also identified tandem xenobiotic-responsive element (XRE) motifs containing the substitution-intolerant core sequence 5'-CACGC-3' at position -126 (GGCACGCCACGCG). That sequence is generally recognized by AhR and AhR nuclear translocator heterodimer,<sup>18</sup> although the flanking sequences are not typical compared with *cyp1a1* XRE motifs<sup>19,20</sup> (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]).<sup>21</sup>

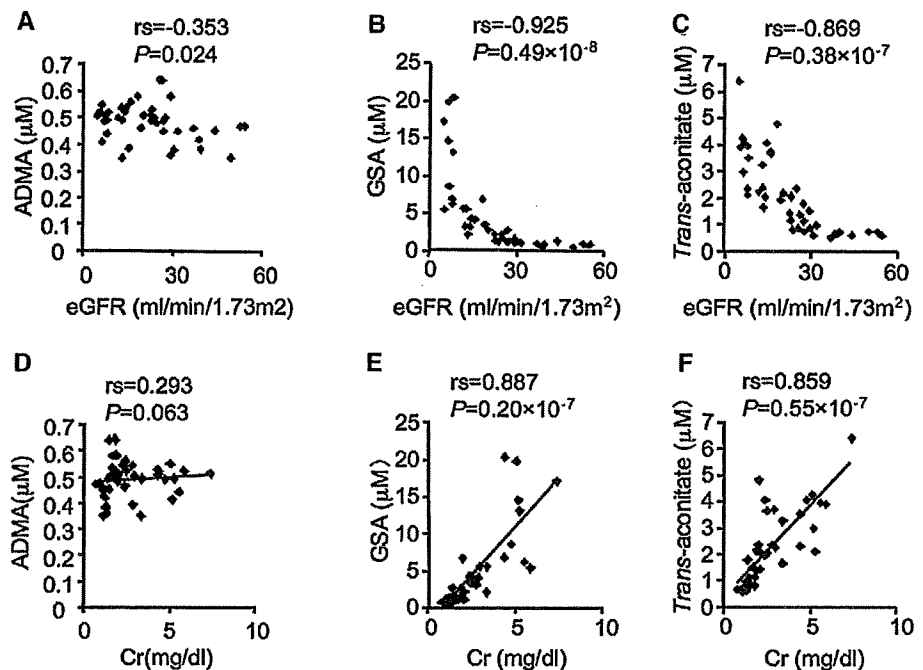


Human *SLCO4C1* promoter activity was increased 1.49-fold ( $-2064$ ) and 1.68-fold ( $-129$ ) by 3-MC compared with controls (Figure 5B). The  $-129$  construct exhibited the highest activity, and this segment contained XRE core motifs. Because AhR can also bind to a structurally divergent range of chemicals,<sup>21</sup> we next screened various compounds. The hepatic hydroxymethyl glutaryl-CoA reductase inhibitor (statin) fluvastatin (2.3-fold at  $10 \mu\text{M}$ ) and pravastatin (1.3-fold at  $30 \mu\text{M}$ ) and atypical AhR ligand flutamide (1.4-fold at  $10 \mu\text{M}$ ) 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,<sup>22</sup> 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 T<sub>3</sub>, 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.

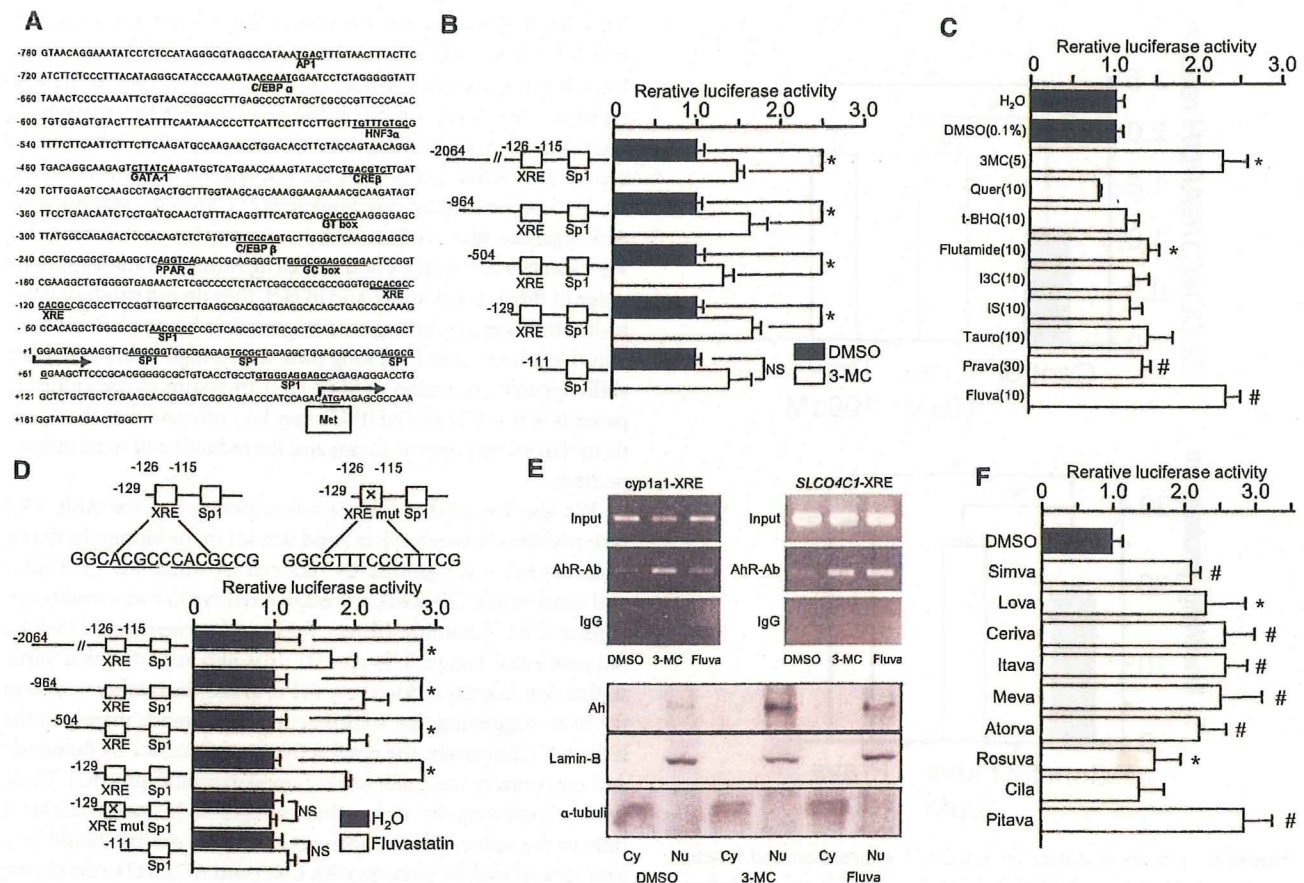


**Figure 4.** Relation between uremic toxins and eGFR as well as plasma creatinine in 41 patients with CKD is shown: (A through C) Correlations between eGFR and the plasma ADMA (A), GSA (B), and *trans*-aconitate (C) in patients with CKD. (D through F) Concentrations between plasma creatinine (Cr) and 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.<sup>23,24</sup> In addition, pravastatin has been reported to modulate DDAH activity and modulate ADMA concentration.<sup>25</sup> 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

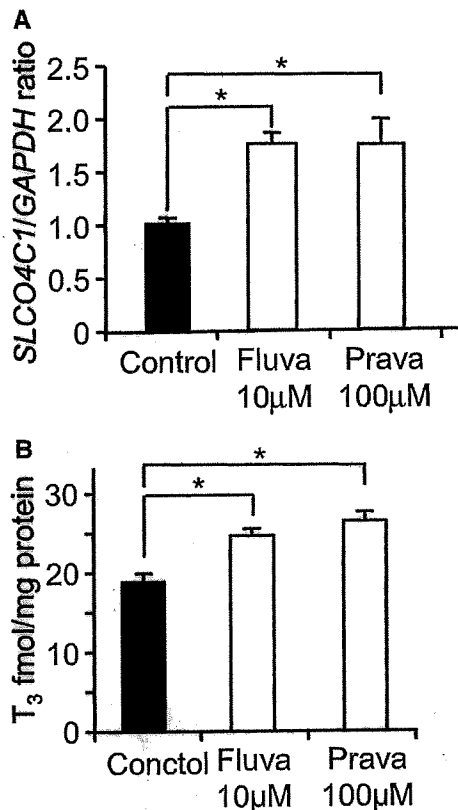


**Figure 5.** Transcriptional analysis and ligand screening are shown. (A) The 5' region of human *SLCO4C1*. Potential *cis*-acting sequences are indicated. Met, first methionine. (B) Promoter activity of human *SLCO4C1*. Deletion constructs of the human *SLCO4C1* promoter region were analyzed with 3-MC (5  $\mu$ M). \* $P$  < 0.05 ( $n$  = 3 to 4 per group). (C) Enhancement of promoter activity of human *SLCO4C1* with various compounds (concentration as indicated,  $\mu$ 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 H<sub>2</sub>O ( $n$  = 3 to 4 per group). (D) Effect of fluvastatin (10  $\mu$ 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  $\mu$ M) or fluvastatin (10  $\mu$ 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  $\alpha$ -tubulin antibodies. Cy, cytosolic fraction; Nu, nuclear fraction. (F) Enhancement of human *SLCO4C1* promoter activity with various statins (10  $\mu$ 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.<sup>2,3</sup> GSA accumulation causes various harmful effects, such as inhibition of platelet aggregation hemolysis and convulsions.<sup>26</sup> 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.<sup>6,7</sup> *Trans*-aconitate, known as anti-feedant in brown plant hoppers,<sup>27</sup> is an inhibitor of aconitase and inhibits the TCA cycle<sup>16</sup>; 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.<sup>28</sup> *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  $\mu$ M) or pravastatin (100  $\mu$ M;  $n = 3$  per group). (B) The uptake of T<sub>3</sub> by ACHN cells treated with fluvastatin (10  $\mu$ M) and pravastatin (100  $\mu$ 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.<sup>22</sup> 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.<sup>22,29</sup> Recently, the relationship between statin administration and ADMA was examined in humans. The serum level of ADMA in metabolic syndrome was reduced by fluvastatin.<sup>30</sup> 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),<sup>5</sup> 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.<sup>31</sup> Among these CYP enzymes, *cyp1a1* is important in the metabolism of carcinogens such as dioxin and halogenated

aromatic hydrocarbons.<sup>31</sup> 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.<sup>32</sup> Clinically, various weak AhR ligands, such as flutamide, omeprazole, and atorvastatin, were identified<sup>32</sup> 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 upregulated 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.<sup>32</sup> 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.<sup>4</sup> Increase of citrulline and trimethyl N-oxide,<sup>33</sup> 3-methylhistidine,<sup>34</sup> N,N-dimethylglycine,<sup>35</sup> and allantoin<sup>36</sup> and decrease of carnitine,<sup>37</sup> Trp, and Tyr<sup>38</sup> were also reported in renal failure.

On the other hand, increase of *trans*-aconitate, 4-acetylbutyrate, hexanoate, argininosuccinate,  $\alpha$ -amino adipate, and pipercolate and decrease of desethylatrazine 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.

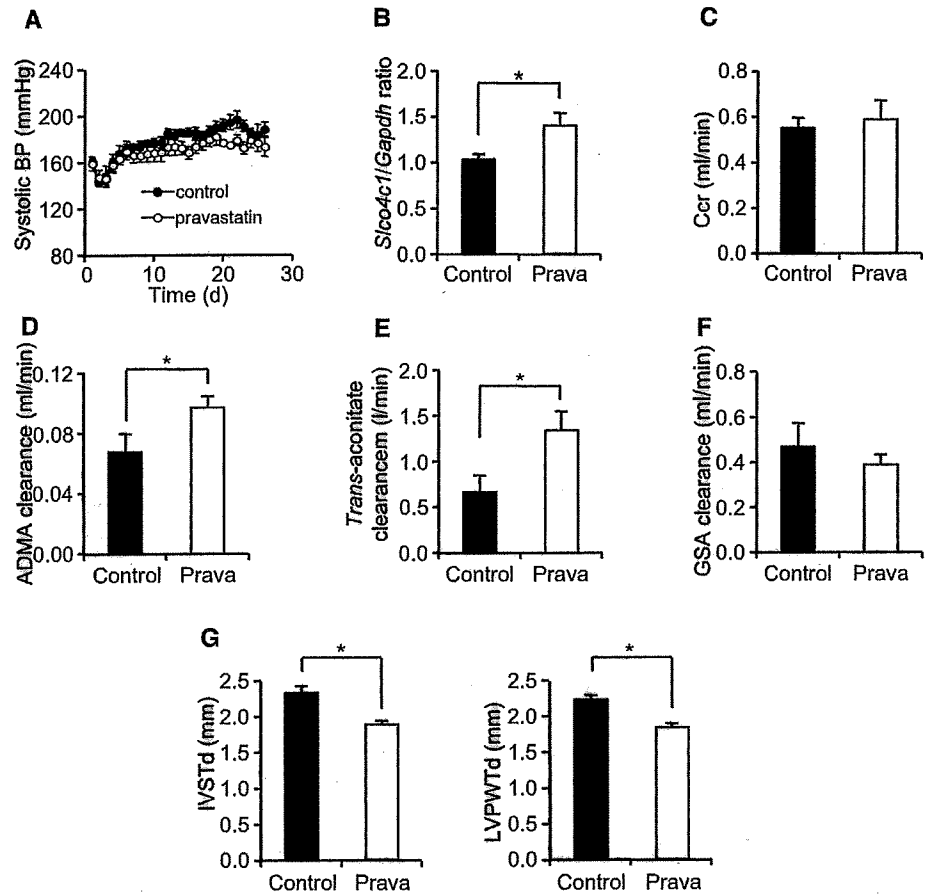
## CONCISE METHODS

### 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 SLCO4C1<sup>10</sup> was inserted into the pGEM-sgt2-5pr-mut plasmid containing kidney-specific sgt2 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). \* $P < 0.05$ .

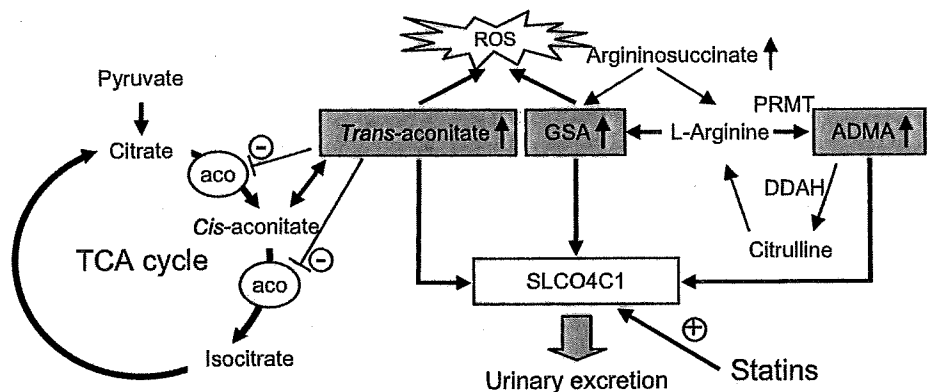
motor.<sup>11</sup> The linear purified plasmid was injected into the pronuclei of fertilized oocytes of Wistar rats. Pups were analyzed for the genomic integration by Southern blotting and by PCR amplification of tail DNA using the following primers: Forward (mouse *splt2*) 5'-tccccaccctctgtt-tccagctatgt-3' and reverse (human *SLCO4C1*) 5'-acgcgatctgcagaatt-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,<sup>39</sup> 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.<sup>10</sup> 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).<sup>40</sup>

### 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.<sup>41</sup>

### CE-MS Method for Metabolome Analysis

A comprehensive and quantitative analysis of charged metabolites by CE-MS was performed.<sup>13</sup> 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 formula<sup>42</sup>  $eGFR \text{ (ml/min per } 1.73 \text{ m}^2) = 175 \times \text{creatinine}^{-1.154} \times \text{age}^{-0.203} \times 0.742 \text{ (if female)} \times 0.741$ .

### Measurement of Reactive Oxygen Species

The free radical formation within the human kidney proximal cell line HK-2 evoked by *trans*-aconitine (100  $\mu$ 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)<sup>43</sup> 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  $\mu$ g of *Renilla* Luciferase Reporter Vector PhRL-TK (Promega) as well as co-transfection with AhR and AhR nuclear translocator expression vector.<sup>18</sup> 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  $\alpha$  (bezafibrate, fenofibrate, clofibrate, and LTB<sub>4</sub>) did not affect the SLCO4C1 transcription (data not shown).

### ChIP Assay

ChIP assays were performed as described previously.<sup>44</sup> 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'-CTAAGTATGGTGGAGGAAAGGGTG-3'). Nuclear and cytoplasmic fraction extracts were prepared and Western blotting was performed as described previously<sup>39</sup> using antibodies against AhR, Lamin B (Santa Cruz Biotechnology, Santa Cruz, CA), and  $\alpha$ -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.

### ACKNOWLEDGMENTS

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### DISCLOSURES

None.

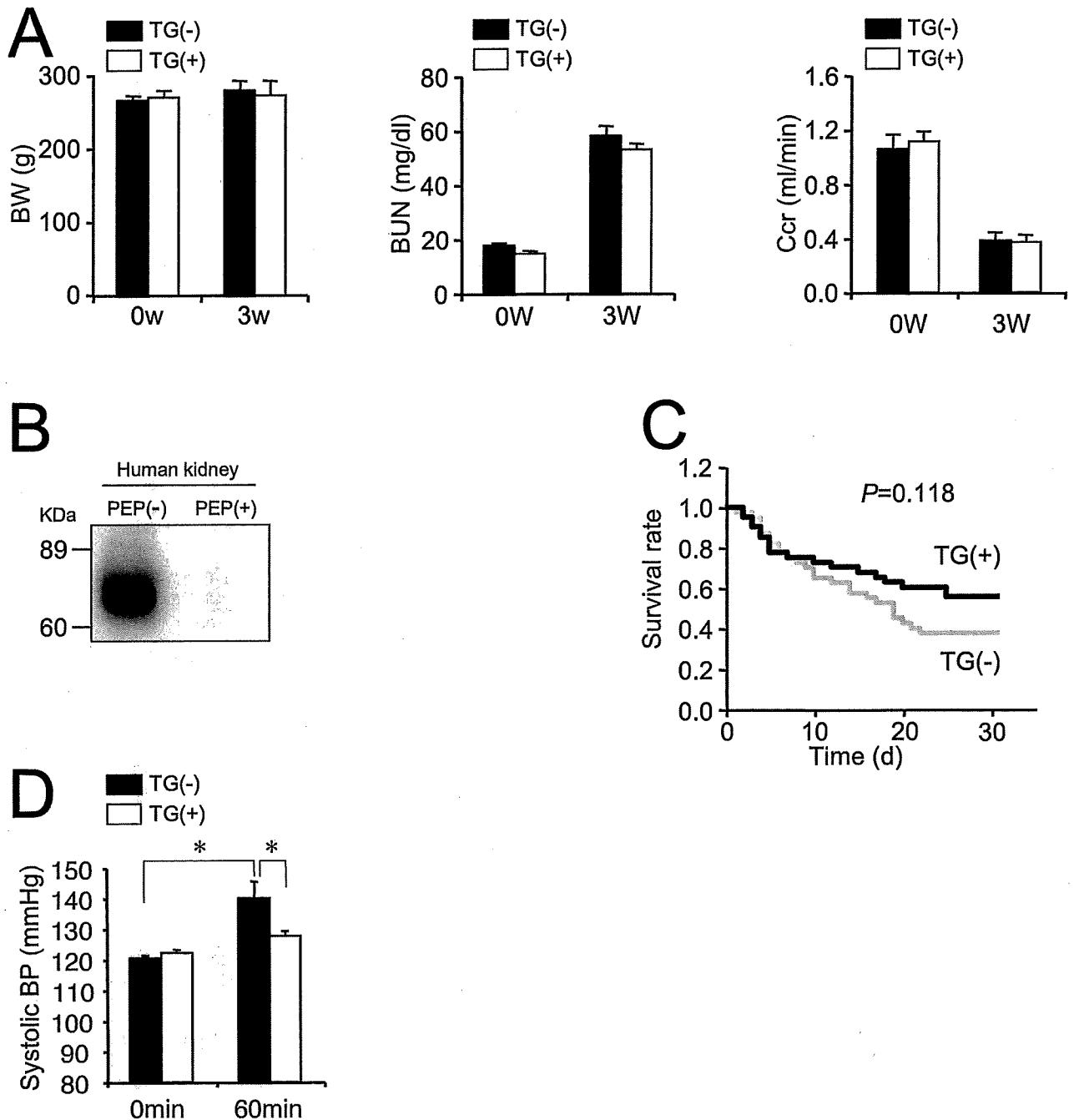
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- See related editorial, "Harnassing Transporters to Clear Uremic Toxins," on pages 2483–2484.
- Supplemental information for this article is available online at <http://www.jasn.org/>.

# Supplementary Figure 1

Toyohara T. et al.



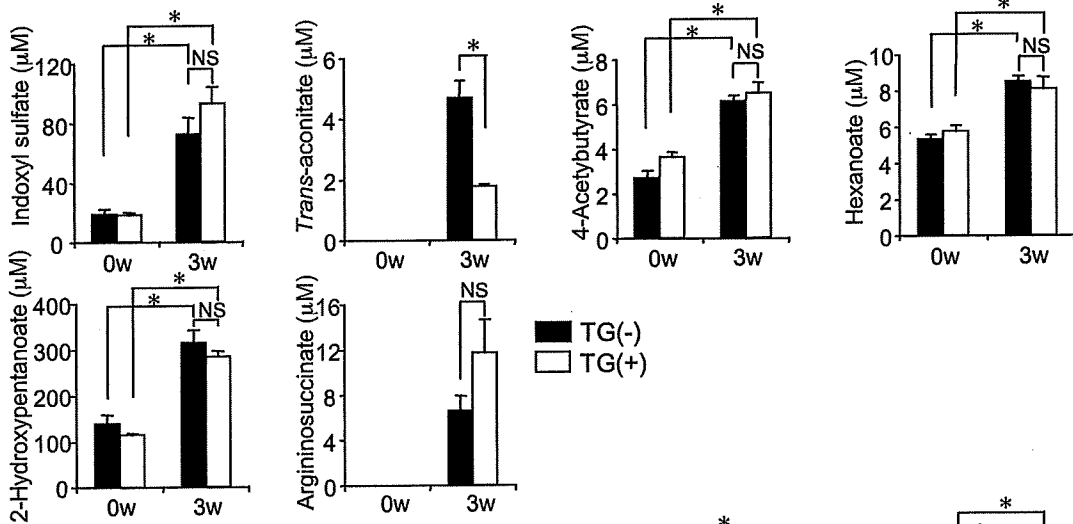
**Supplementary Figure 1** Characterization of TG rats. (A) Body weight and biochemical examinations of nephrectomized TG rats ( $n=16-20$  per group). (B) Western blotting with whole human kidney protein using antibody against human SLCO4C1. A single band was abolished by peptide absorption, suggesting the specificity of the antibody. (C) Survival rate of TG(-) rat ( $n=41$ ) and TG(+) rat ( $n=40$ ) after 5/6 nephrectomy. Kaplan-Meier curves and log-rank test were used to analyze the mortality rates.  $P=0.118$  by log-rank test.

(D) Blood pressure after intraperitoneal-injection of *trans*-aconitase (400mg/kg) to TG(-) rat and TG(+) rat ( $n=3$  per group).

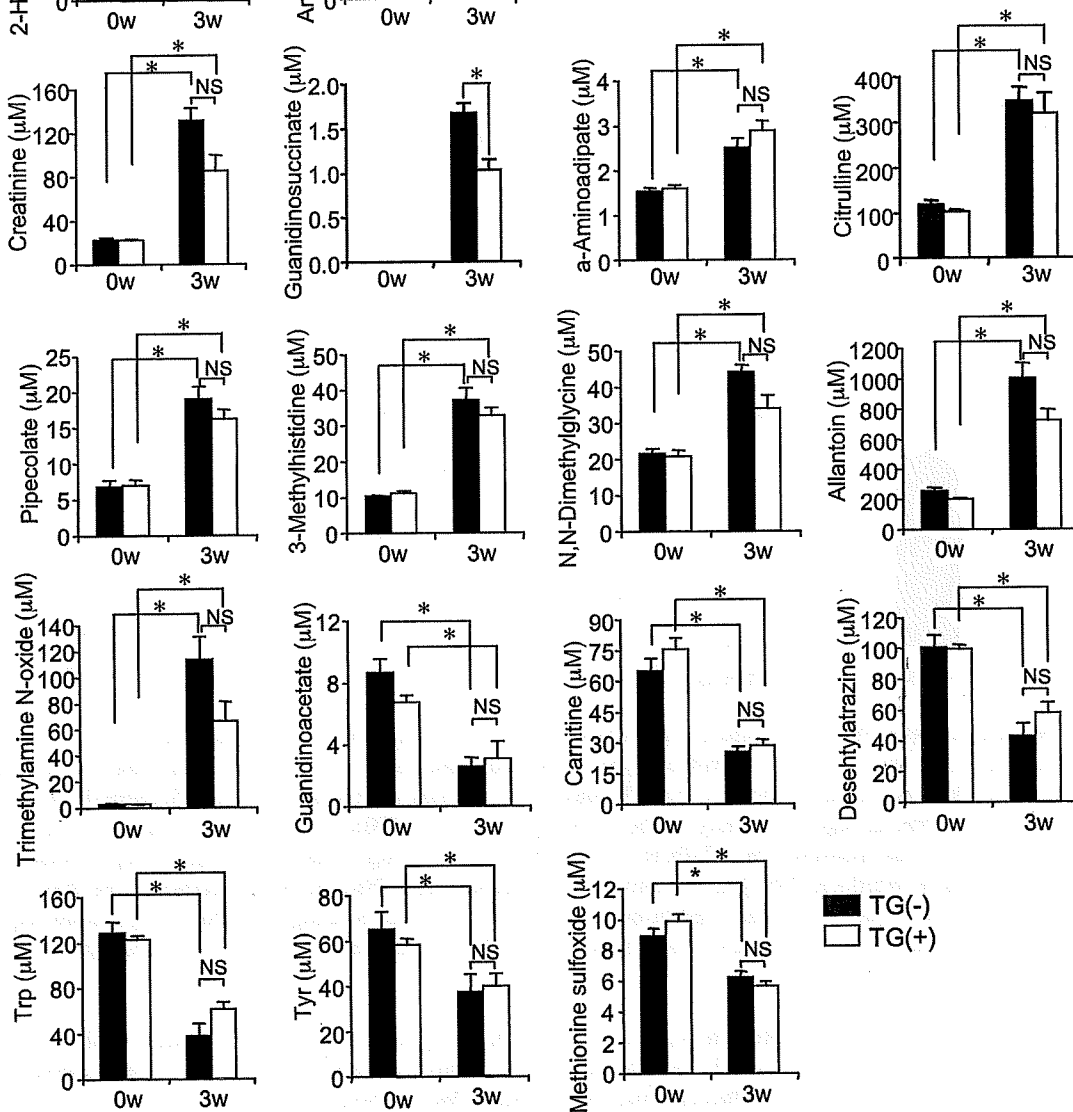
# Supplementary Figure 2

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anion

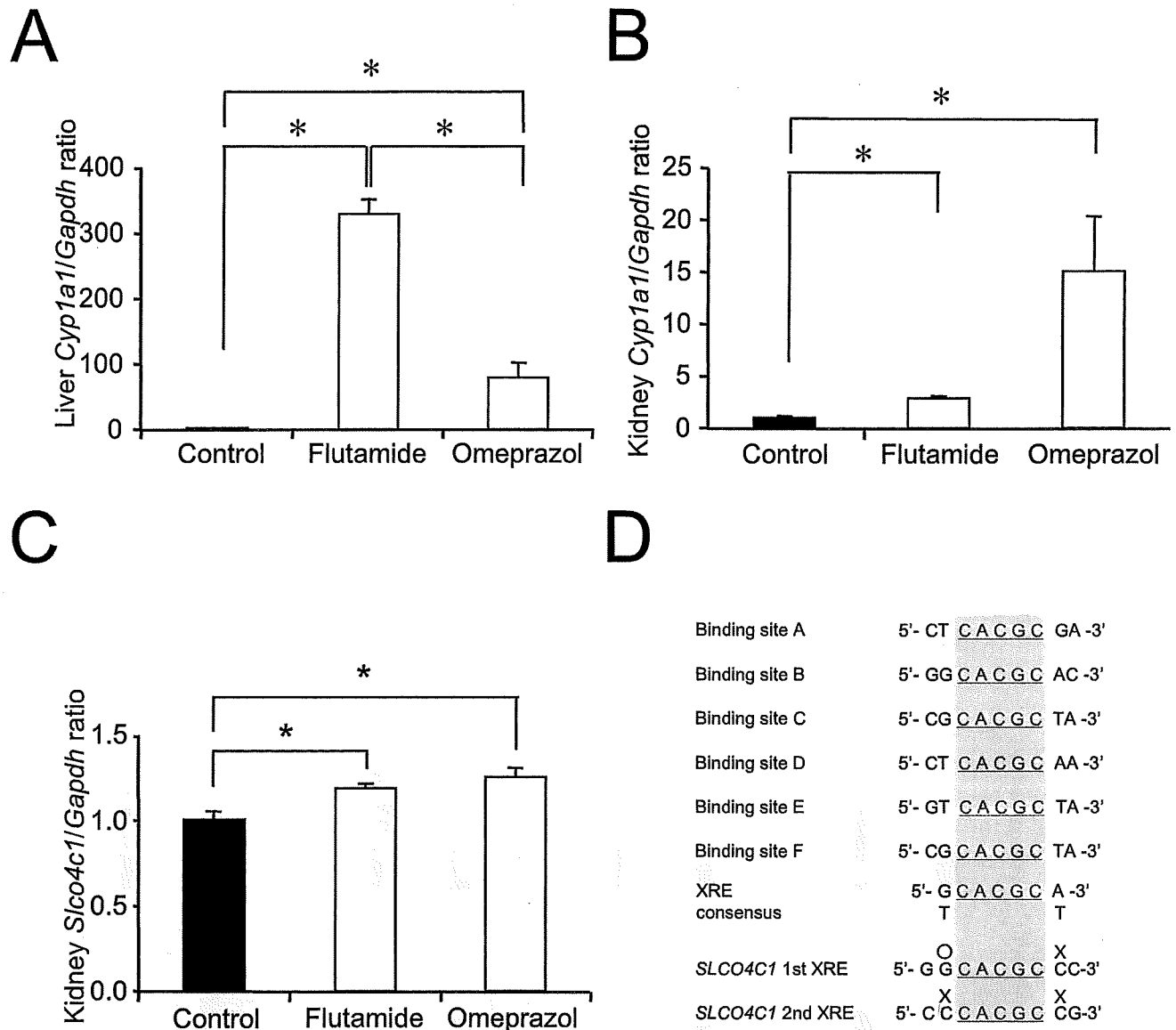


cation



**Supplementary Figure 2** Metabolome analysis of uremic toxins measured by CE-MS. Anionic and cationic compounds that were significantly increased or decreased after nephrectomy were nominated (all analyzed data are in Supplementary Tables 1 - 4). Note that, among these, the plasma levels of GSA and *trans*-aconitate were significantly decreased in TG(+)*N*x rats 3 weeks after nephrectomy. Closed column; TG(-) rats, open column; TG(+)*N*x rats. \**P*<0.05. (*n*=4-5 per group).





**Supplementary Figure 3** Different Induction of *cyp1a1* transcription by atypical AhR ligands. QT-PCR analysis of rat liver *cyp1a1* (A), kidney *cyp1a1* (B) and kidney *slco4c1* (C) after administration of flutamide (15.6 mg/kg/day) or omeprazole (125 mg/kg/day). Compounds were administered to Sprague-Dawley rats in corn oil by oral gavage once daily in the morning (Coe et al, 2006). Three days after administration, the mRNA level was measured by QT-PCR. \* $P < 0.05$  ( $n = 3$  per group). (D) Alignment of representative XRE motifs (Wu & Whitlock, 1993) and putative consensus sequence (Nioi & Hayes, 2004). Note that, although the XRE core sequence is conserved in human SLCO4C1, its surrounding 5'- and 3'-nucleotide sequences were not identical to the other XRE consensus motifs. O, consensus pattern; X, non-consensus pattern.

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Wu L, Whitlock JP, Jr. (1993) Mechanism of dioxin action: receptor-enhancer interactions in intact cells. *Nucleic Acids Res* 21: 119-125





