Table 2 Hematocrit levels and prevalence of anemia by clinical characteristics

	All ($N = 94,602$)			Men ($N = 39,754$)			Women ($N = 54,848$)		
	Number	Hematocrit (%)	Anemia, number (prevalence)	Number	Hematocrit (%)	Anemia, number (prevalence)	Number	Hematocrit (%)	Anemia, number (prevalence)
BMI (kg/m^2)									
≥26	24367	42.3 ± 4.0 (ref)	664 (2.7)	10422	45.5 ± 3.0 (ref)	317 (3.0)	13945	40.0 ± 2.8 (ref)	347(2.5)
24–26	20942	41.9 ± 4.0*	921 (4.4)	96651	44.9 ± 3.0*	489 (5.1)	11281	39.4 ± 2.9*	432 (3.8)
22–24	22287	41.3 ± 4.1*	1325 (5.9)	9645	44.4 ± 3.2*	726 (7.5)	12642	38.9 ± 2.9*	599(4.7)
<22	26241	40.3 ± 4.0*	2429 (9.3)	9754	43.4 ± 3.6*	1471(15.0)	16487	38.5 ± 3.0*	958(5.8)
ANOVA		$P < 0.0001$	$P < 0.0001$		$P < 0.0001$	$P < 0.0001$		$P < 0.0001$	$P < 0.0001$
Age (years)									
20–29	5423	42.8 ± 4.3 (ref)	53(1.0)	2773	46.1 ± 2.7 (ref)	32(1.2)	2650	39.4 ± 2.6 (ref)	21(0.8)
30–39	11802	41.9 ± 4.7*	294 (2.5)	5746	46.7 ± 2.8*	99 (1.7)	6056	38.3 ± 3.0*	195(3.2)
40–49	17612	41.3 ± 4.7*	671 (3.8)	7723	45.3 ± 2.9*	210 (2.7)	9889	38.2 ± 3.3*	461(4.7)
50–59	19996	41.6 ± 3.7*	811 (4.1)	7684	44.7 ± 3.0*	340 (4.4)	12312	39.7 ± 2.7#	471(3.8)
60–69	22446	41.5 ± 3.6*	1306 (5.8)	9035	44.0 ± 3.2*	833 (9.2)	13411	39.7 ± 2.7§	473(3.5)
≥70	17323	40.5 ± 3.8*	2320 (13.4)	6793	42.6 ± 3.8*	1542 (22.7)	10530	39.2 ± 3.1	778(7.4)
ANOVA		$P < 0.0001$	$P < 0.0001$		$P < 0.0001$	$P < 0.0001$		$P < 0.0001$	$P < 0.0001$
Estimated GFR (ml/min per 1.73 m^2)									
≥90	25258	41.4 ± 4.4 (ref)	1084 (4.3)	10709	45.0 ± 3.0 (ref)	459 (4.3)	14549	38.7 ± 3.1 (ref)	625 (4.3)
60–89	54042	41.7 ± 4.0*	2836 (5.3)	24100	44.6 ± 3.3*	1741 (7.2)	29942	39.4 ± 2.9*	29942 (3.7)
45–59	13287	40.8 ± 3.8*	1115 (8.4)	4360	43.6 ± 3.8*	642 (14.7)	8927	39.4 ± 2.9*	473 (5.3)
30–44	1829	39.6 ± 4.0*	331 (18.1)	524	41.9 ± 4.5*	174 (33.2)	1305	38.7 ± 3.4	157 (12.0)
15–29	151	37.4 ± 5.0*	60 (39.7)	47	39.2 ± 5.9*	27 (57.5)	104	36.6 ± 4.5*	33 (31.7)
<15	35	31.5 ± 4.9*	29 (82.9)	14	31.6 ± 4.8*	13 (92.9)	21	31.5 ± 5.0*	16(76.2)
ANOVA		$P < 0.0001$	$P < 0.0001$		$P < 0.0001$	$P < 0.0001$		$P < 0.0001$	$P < 0.0001$

* <0.0001, # <0.05, § <0.0005

(eGFR 60–89 ml/min per 1.73 m^2), 5.9% (eGFR 45–59 ml/min per 1.73 m^2), 12.3% (eGFR 30–44 ml/min per 1.73 m^2), 32.7% (eGFR 15–29 ml/min per 1.73 m^2), and 81.0% (eGFR <15 ml/min per 1.73 m^2) when JSDT anemia criteria were applied.

Kidney function and the odds ratio of anemia

We performed multiple logistic analyses adjusted for older age (70 years and older) and BMI category to further assess the effect of decreased kidney function on anemia. Lower eGFR was found to be significantly associated with higher prevalence of anemia below eGFR of 90 ml/min per 1.73 m^2 in men and of 45 ml/min per 1.73 m^2 in women (Fig. 2). The odds ratios (ORs) of eGFR categories (ref, eGFR ≥90 ml/min per 1.73 m^2) overall, in men, and in women were as follows: eGFR 60–89 ml/min per 1.73 m^2: 1.150 (1.067–1.240, $P = 0.003$), 1.536 (1.374–1.717, $P < 0.0001$), and 0.857 (0.772–0.950, $P < 0.0001$); eGFR 45–59 ml/min per 1.73 m^2: 1.526 (1.385–1.681, $P < 0.0001$), 2.278 (1.979–2.622, $P < 0.0001$), and 1.076 (0.940–1.233, $P = 0.2885$); eGFR 30–44 ml/min per 1.73 m^2: 2.976 (2.564–3.454, $P < 0.0001$), 5.117 (4.072–6.431, $P < 0.0001$), and 2.265 (1.843–2.783, $P < 0.0001$); eGFR 15–29 ml/min per 1.73 m^2: 11.346 (7.909–16.276, $P < 0.0001$), 24.404 (12.710–46.857, $P < 0.0001$), and 8.234 (5.269–12.867, $P < 0.0001$); and eGFR ≤15 ml/min per 1.73 m^2: 104.250 (41.632–261.049, $P < 0.0001$), 288.024 (36.039–2301.922, $P < 0.0001$), and 65.386 (23.265–183.767, $P < 0.0001$). The OR of older age (over 70 years) was 2.772 (2.597–2.959, $P < 0.0001$) overall, 3.850 (3.531–4.198, $P < 0.0001$) in men, and 1.698 (1.530–1.884, $P < 0.0001$) in women. Additionally, the ORs of BMI categories (ref. BMI ≥26 kg/m^2) overall, in men, and in women were as follows: BMI 24–26 kg/m^2: 1.565 (1.412–1.735, $P < 0.0001$), 1.552 (1.339–1.798, $P < 0.0001$), and 1.580 (1.367–1.826, $P < 0.0001$); BMI 22–24 kg/m^2: 2.159 (1.960–2.377, $P < 0.0001$), 2.305 (2.007–2.648, $P < 0.0001$), and 1.959 (1.710–2.244, $P < 0.0001$); BMI <22 kg/m^2: 3.571 (3.264–3.907 $P < 0.0001$), 4.543 (3.991–5.171, $P < 0.0001$), and 2.466 (2.172–2.800, $P < 0.0001$).

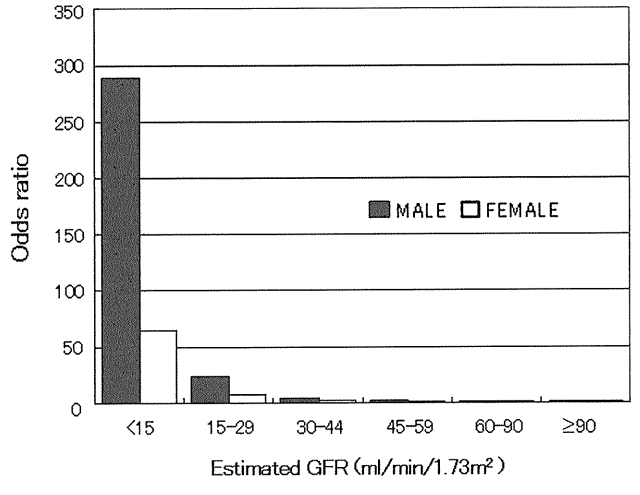

Fig. 2 Odds ratio of anemia by sex, adjusted for body mass index category and older age (>70 years) according to estimated glomerular filtration rate category in both sexes. Reference is eGFR ≥90 ml/min per 1.73 m^2

Prevalence of stage 3–5 CKD complicated with anemia

The result of the present study showed that 10% of subjects with stage 3–5 CKD were complicated with anemia. Since it has been estimated that there are 10,000,000 Japanese people with stage–3–5 CKD by using a new Japanese equation: eGFR (ml/min per 1.73 m^2) = 194 × serum creatinine$^{1.094}$ × age$^{0.287}$ × 0.739 (if female) [18], there could be as many as 1,000,000 Japanese people with stage 3–5 CKD complicated with anemia.

Discussion

Anemia is often associated with decreased eGFR. However, previous reports have suggested that the relationship between decreased kidney function and anemia varies across countries and races [15, 16, 23]. In the present study, which was conducted among a general Japanese population, the effect of decreased kidney function on anemia was significantly prevalent below eGFR of 90 ml/min per 1.73 m^2 in men and 45 ml/min per 1.73 m^2 in women.

As the previous study demonstrated [12], the distribution of eGFR among the general Japanese population is shifted to the lower side compared with that of the general US population [17]: the mean eGFR value was approximately 79 ml/min per 1.73 m^2 in our cohort, while it is reported to be 93 ml/min per 1.73 m^2 in the USA [17]. The higher incidence of aged subjects might be responsible for the lower eGFR value in Japan. Alternatively, the normal kidney function of the Japanese population might be fundamentally less than that of Caucasian populations due to the relatively smaller size of kidney and lower intake of protein. Regardless of its cause, a cutoff value of eGFR for clinical relevance is yet to be determined for the Japanese population. Some researchers have argued that it would be approximately 50 ml/min per 1.73 m^2 since the risk of end-stage renal disease (ESRD) increases significantly at this level [25]. In the present study, the OR of anemia increased to more than twice at eGFR values of less than approximately 50 ml/min per 1.73 m^2. According to the present study, the adjusted OR of stage 3 CKD for anemia in the Japanese general population has been shown to be around

two, which is similar to that in the general US population [16]. In terms of risk for complicating anemia, the clinical eGFR value in Japan might be similar to that of the general US population.

In the US population in the Third National Health and Nutrition Examination Survey (NHANES III), it was shown that African–Americans had a significantly higher OR (2.5) for anemia than Caucasians [16]. In another study from Italy conducted among patients whose mean age was about 75 years, the threshold of kidney function as a risk factor of anemia was found to be 30 ml/min per 1.73 m^2, which is lower than that of Japanese and US populations [24]. Although age might be responsible for the difference in the threshold level of kidney function in the Italian study, we found no such difference between subjects 70 years and older, and those under 70 years old (data not shown). Some factors, including differences in the definition of anemia and/or race, may affect this discrepancy.

In addition to racial differences, there might also be gender differences in the rate of complication with anemia at the same degree of kidney function. In the present study, men had a higher incidence and OR for anemia compared with women at eGFR values below 60 ml/min per 1.73 m^2; this is consistent with the previous report by Hsu et al. [25]. Differences in the cause of CKD between genders [26] and the effect of sex hormones on erythrogenesis might be responsible for this gender difference [27, 28].

The combination of anemia and CKD is reported to have a significant impact on survival compared with either anemia alone or CKD alone [29]. Since anemia has been identified not only as a nonclassical cardiovascular risk factor but also as a progressive factor in decreasing kidney function, anemia might play a significant role in the association between CKD and CVD. Accordingly, intervention for anemia could be an effective approach to prevent CVD in CKD subjects. However, large randomized intervention studies [30, 31] and a meta-analysis [32] have shown a slight but significant benefit of lower hemoglobin levels; it would thus be better to maintain these lower levels rather than attempt to improve outcome by achieving higher hemoglobin levels in CKD patients. Since the higher hemoglobin target group showed itself to have a higher risk of poorly controlled blood pressure [32], the clinical benefits of correction of anemia via an erythropoiesis-stimulating agent should be determined under strict control of blood pressure. Considering the substantial number of patients complicated with CVD and related death before starting hemodialysis therapy, intervention during ESRD might be too late to effectively prevent CVD. The incidence of anemia appears to increase from an eGFR of less than 60 ml/min per 1.73 m^2, as shown in previous studies [16] as well as in the present study. Therefore, intervention for anemia in the early stages of CKD could be an effective method of preventing CVD among CKD subjects.

In Japan, incidence of CKD is predicted to be much higher than that in the US population [12, 17]. Furthermore, it will increase since the number of elderly people is predicted to increase in Japan, at least during the next two decades. According to the present study, an association of kidney function with anemia was similar to that in the US population. Therefore, it is critical to screen CKD subjects for anemia.

The present study has a number of important limitations. First, we were unable to identify any causal association between decreased kidney function and anemia due to the cross-sectional design of the study. It was not clear how long-term CKD contributes to anemia at each CKD stage. We cannot exclude the possibility that other factors such as iron deficiency, malnutrition, and chronic disease might affect anemia. Second, one-third of the total cohort was excluded because of lack of data for Scr and Ht. It is possible that those with known kidney diseases and/or comorbid individuals are selected. However, the total number of subject is more than 90,000 and therefore it is subtle as a community-based cohort. Third, the results might vary according to the definition of anemia. The assessment of anemia by hematocrit may not be always precise and may be affected by volume status. Previous studies investigating the relationship between renal function and anemia have used the World Health Organization (WHO) criteria to define anemia [15, 16]. The WHO defines anemia as hemoglobin concentration of less than 12 g/dl for women and less than 13 g/dl for men. However, these criteria have physiological correlates in younger individuals. Therefore, it has been suggested that it might be inappropriate to apply these criteria to the present cohort, which included a substantially high number of older subjects [33]. Thus, it might be preferable to use the definition of anemia, which takes both age and sex into account [20, 21].

In conclusion, the threshold level of kidney function, below which there is an increased risk of more than twice for complicating anemia, was found to be an eGFR of approximately 50 ml/min per 1.73 m^2 in a general Japanese population. Therefore, there is expected to be a substantial number of CKD subjects with anemia who could have a higher risk for CVD as well as ESRD. Further information is needed to determine how and when intervention should be initiated in patients with both CKD and anemia.

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SLCO4C1 Transporter Eliminates Uremic Toxins and Attenuates Hypertension and Renal Inflammation

Takafumi Toyohara,* Takehiro Suzuki,* Ryo Morimoto,* Yasutoshi Akiyama,* Tomokazu Souma,* Hiromi O. Shiwaku,* Yoichi Takeuchi,* Eikan Mishima,* Michiaki Abe,* Masayuki Tanemoto,* Satohiro Masuda,† Hiroaki Kawano,‡ Koji Maemura,‡ Masaaki Nakayama,§ Hiroshi Sato,* Tsuyoshi Mikkaichi,‖ Hiroaki Yamaguchi,‖ Shigefumi Fukui,¶ Yoshihiro Fukumoto,¶ Hiroaki Shimokawa,¶ Ken-ichi Inui,† Tetsuya Terasaki,** Junichi Goto,‖ Sadayoshi Ito,* Takanori Hishinuma,†† Isabelle Rubera,‡‡ Michel Tauc,‡‡ Yoshiaki Fujii-Kuriyama,§§ Hikaru Yabuuchi,‖‖ Yoshinori Moriyama,¶¶ Tomoyoshi Soga,*** and Takaaki Abe*†††‡‡‡

*Division of Nephrology, Endocrinology, and Vascular Medicine, §Research Division of Dialysis and Chronic Kidney Disease, ¶Department of Cardiovascular Medicine, and ‡‡‡Department of Clinical Biology and Hormonal Regulation, Tohoku University Graduate School of Medicine, Sendai, Japan; †Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Kyoto, Japan; ‡Department of Cardiovascular Medicine, Nagasaki University School of Medicine, Nagasaki, Japan; ‖Department of Pharmaceutical Sciences, Tohoku University Hospital, Sendai, Japan; **Division of Membrane Transport and Drug Targeting and ††Division of Pharmacotherapy, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan; ‡‡CNRS-FRE3093, University of Nice-Sophia Antipolis, Parc Valrose, Nice Cedex 2, France; §§Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan; ‖‖GenoMembrane Inc., Yokohama, Japan; ¶¶Department of Membrane Biochemistry, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan; ***Institute for Advanced Biosciences, Keio University, Tsuruoka, Japan; and †††Division of Medical Science, Tohoku University Graduate School of Biomedical Engineering, Sendai, Japan

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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T.To., T.Su., and R.M. contributed equally to this work.

T.H. is deceased.

Correspondence: Dr. Takaaki Abe, Division of Medical Science, Tohoku University Graduate School of Biomedical Engineering, Sendai 980-8574 Japan. Phone: +81-22-717-7163; Fax: +81-22-717-7168; E-mail: takaabe@mail.tains.tohoku.ac.jp; or Dr. Takehiro Suzuki, Division of Nephrology, Endocrinology, and Vascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seriyo-cho, Aoba-ku, Sendai 980-8574, Japan. Phone: +81-22-717-7163; Fax: +81-22-717-7168; E-mail: suzuki2i@mail.tains.tohoku.ac.jp

All individuals with an estimated GFR (eGFR) <60 ml/min per 1.73 m^2 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 excretion 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 promoter[11] (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).

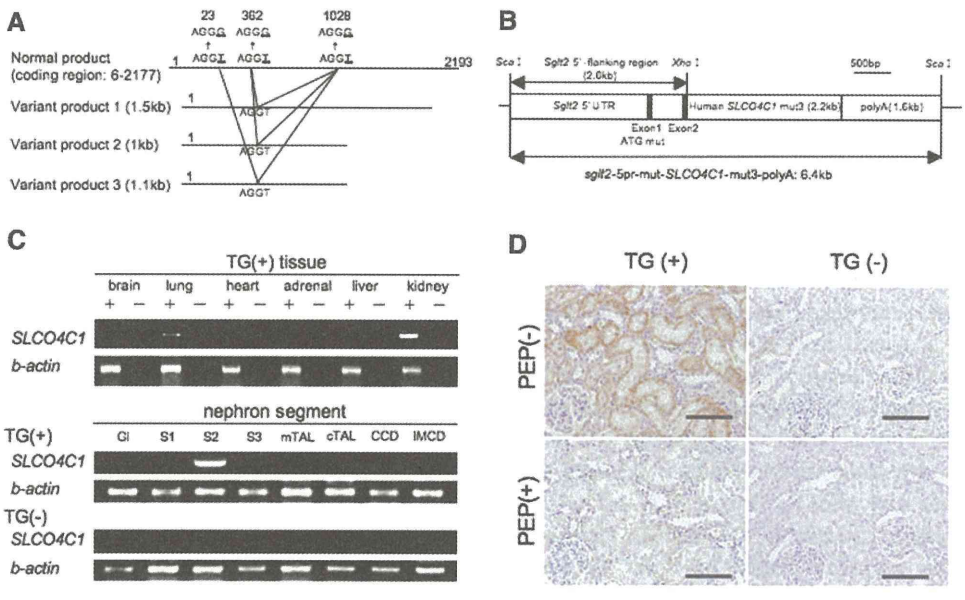

Figure 1. Characterization of human SLCO4C1 TG rats is shown. (A) Three different smaller sizes of mRNA by alternative splicing were found and mutated to avoid unusual splicing (AGGT to AGGG). (B) The mutated human SLCO4C1 cDNA was inserted into a plasmid under the proximal tubule–specific promoter. (C) Expression of human SLCO4C1 in rat organs and microdissected renal tubules examined by reverse transcriptase–PCR. GI, 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 μm.

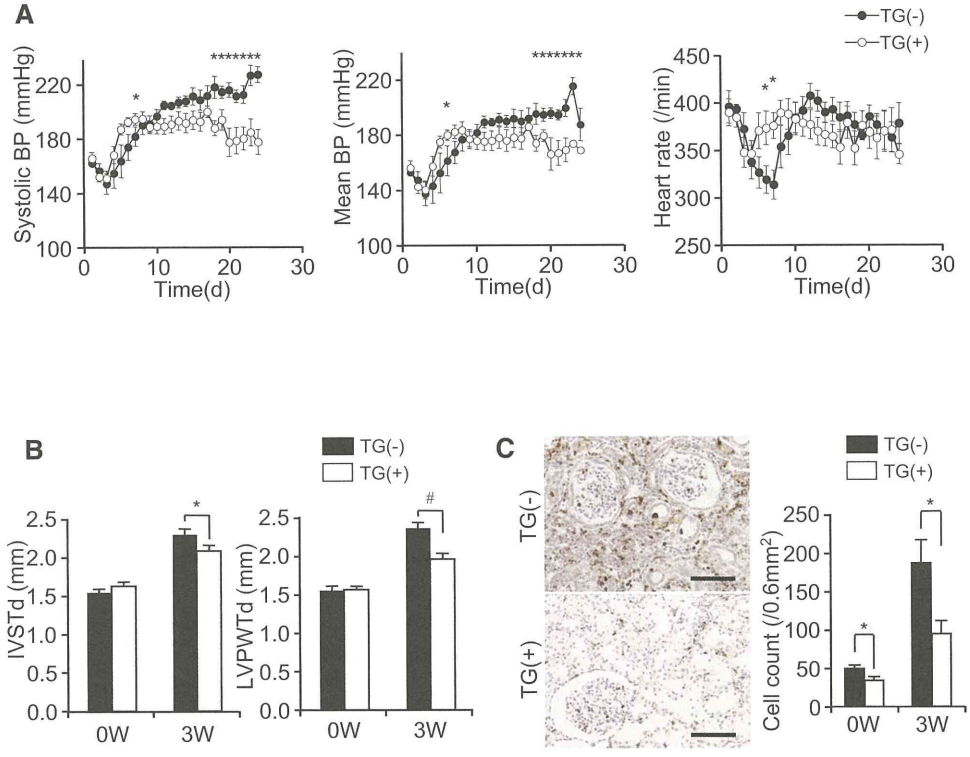

Figure 2. Phenotype of human SLCO4C1 TG rats. (A) BP and heart rate of TG(−)Nx and TG(+)Nx rats. $^{*}P < 0.05$ *versus* TG(−)Nx rats (n = 4 to 6 per group). (B) Thickness of the interventricular septum (IVSTd) and left ventricular posterior wall at end-diastole (LVPWTd) were measured by echocardiogram 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 antihypertensive 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 he accumulation of guanidino compounds (including ADMA and GSA) and several uremic toxins generate oxidative stress, and it causes further renal

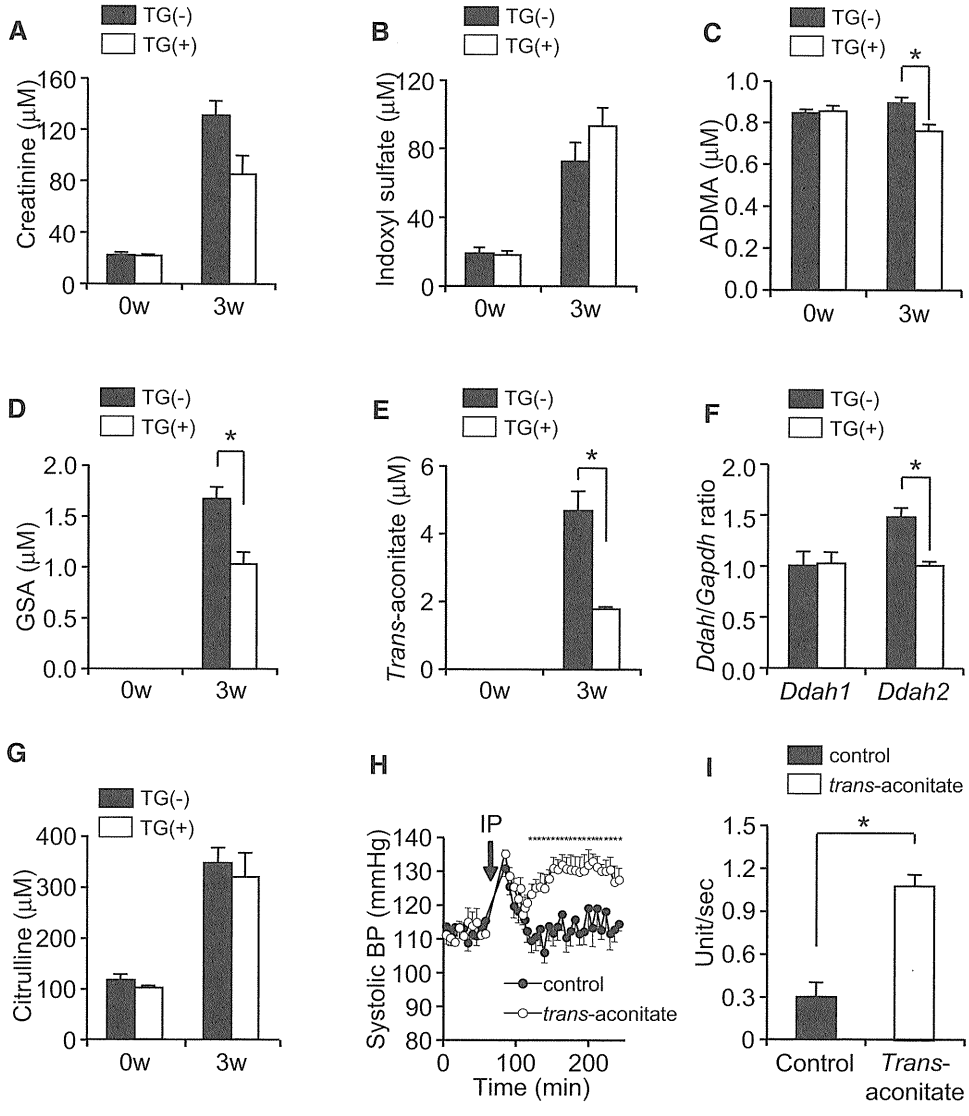

Figure 3. Metabolome analysis and characterization of uremic toxins are shown. (A through E and G) The plasma concentration of creatinine (A), indoxyl sulfate (B), ADMA (C), GSA (D), *trans*-aconitate (E), and citrulline (G) before and 3 wk after five-sixths Nx (n = 4 to 5 per group). (F) The mRNA expression 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[17]; however, the existence in mammals, biologic effects, and the precise role of *trans*-aconitate in renal failure have not been clarified. When *trans*-aconitate was administered to rats intraperitoneally, the BP of injected rats was immediately elevated compared with controls (Figure 3H). This increase of BP was cancelled when *trans*-aconitate was injected into TG(+) rats compared with TG(−) rats, further suggesting the excretion through SLCO4C1 (Supplemental Figure 1D). In addition, *trans*-aconitate significantly induced superoxide production in human kidney proximal tubule cells (Figure 3I).

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

Functional Analysis of SLCO4C1 Promoter and Its Modulation by Statins

We assumed that enhancement of SLCO4C1 in the kidney may facilitate the excretion of uremic toxins and thereby ameliorate the symptoms of CKD. In this scenario, drugs that upregulate SLCO4C1 in the kidney may facilitate excretion of uremic toxins and reduce renal inflammation, decelerating progression of renal damage and entry of hemodialysis. To address this, we isolated the promoter region of human SLCO4C1. Human SLCO4C1 promoter region has a predominant transcription start site located 164 bp upstream of the ATG codon (Figure 5A). Potential *cis*-acting motifs for GATA-1, hepatocyte nuclear factor (HNF)-3α, CCAAT/enhancer-binding protein (C/EBP)α, C/EBPβ, cAMP response element-binding protein (CREB), and peroxisome proliferator–activated receptor α were found. We also identified tandem xenobiotic-responsive element (XRE) motifs containing the substitution-intolerant core sequence 5′-CACGC-3′ at position −126 (GG*CACGCCCACGC*CG). 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 μM) and pravastatin (1.3-fold at 30 μM) and atypical AhR ligand flutamide (1.4-fold at 10 μM) upregulated the SLCO4C1 promoter activity (Figure 5C). Because of the comparable magnitude to 3-MC and its clinical availability, we further focused on statins. Deletion experiments showed that all constructs exerted potent promoter activation, but removal of the XRE core segment or mutation in the XRE core motifs abolished the response to fluvastatin (Figure 5D). Because there are various clinical reports on renoprotective effects of statins,[22] we further examined various statins on human SLCO4C1 transcription. Simvastatin, lovastatin, cerivastatin, 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.

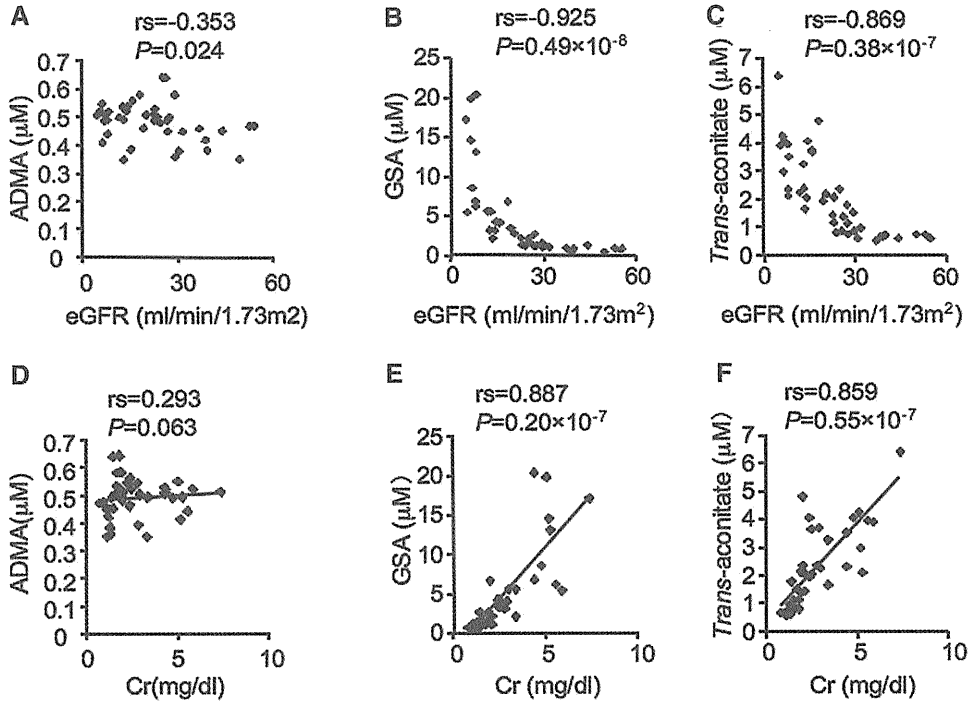

Figure 4. Relation between uremic toxins and eGFR as well as plasma creatinine in 41 patients with CKD is shown. (A through C) Correlations between eGFR and the plasma ADMA (A), GSA (B), and *trans*-aconitate (C) in patients with CKD. (D through F) Concentrations between plasma creatinine (Cr) and plasma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

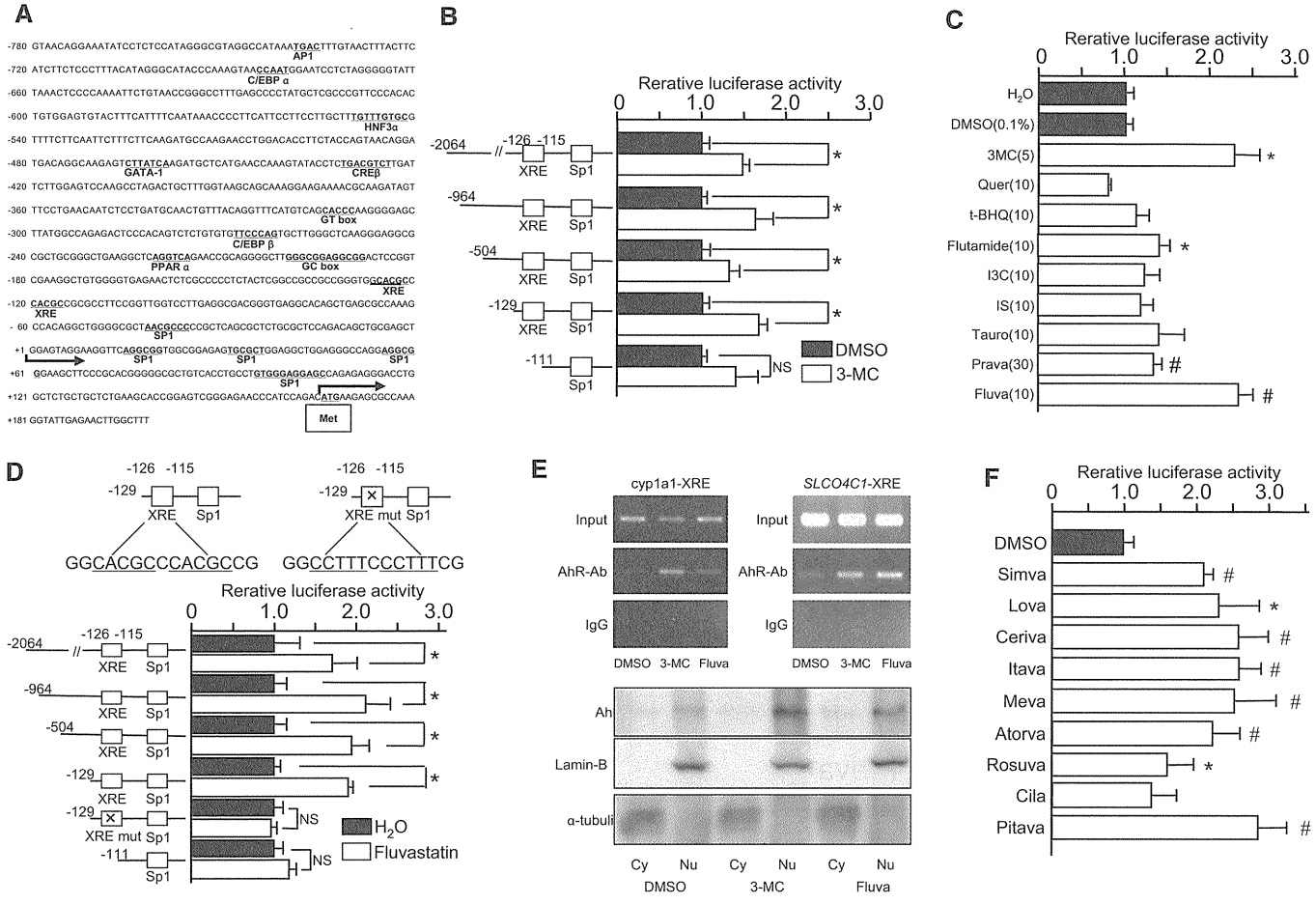

Figure 5. Transcriptional analysis and ligand screening are shown. (A) The 5′ region of human SLCO4C1. Potential *cis*-acting sequences are indicated. Met, first methionine. (B) Promoter activity of human SLCO4C1. Deletion constructs of the human SLCO4C1 promoter region were analyzed with 3-MC (5 μM). $^{*}P < 0.05$ (n = 3 to 4 per group). (C) Enhancement of promoter activity of human SLCO4C1 with various compounds (concentration as indicated, μM). 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_2O (n = 3 to 4 per group). (D) Effect of fluvastatin (10 μM) 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 μM) or fluvastatin (10 μM), 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 μM) 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 cycle[16]; 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-

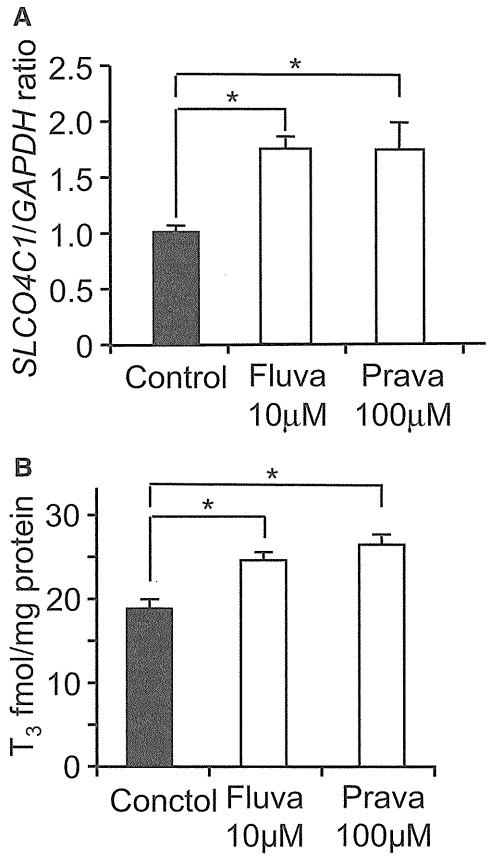

Figure 6. Effects of statins on SLCO4C1 expression and function *in vitro*. (A) Real-time PCR of SLCO4C1 in ACHN cells with fluvastatin (10 μM) or pravastatin (100 μM; $n = 3$ per group). (B) The uptake of T_3 by ACHN cells treated with fluvastatin (10 μM) and pravastatin (100 μM). *$P < 0.05$ ($n =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

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 identified[32] 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.[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 allantoin[36] and decrease of carnitine,[37] Trp, and Tyr[38] were also reported in renal failure.

On the other hand, increase of *trans*-aconitate, 4-acetylbutyrate, hexanoate, argininosuccinate, α-aminoadipate, and pipecolate 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[10] was inserted into the pGEM-sglt2–5pr-mut plasmid containing kidney-specific sglt2 pro-

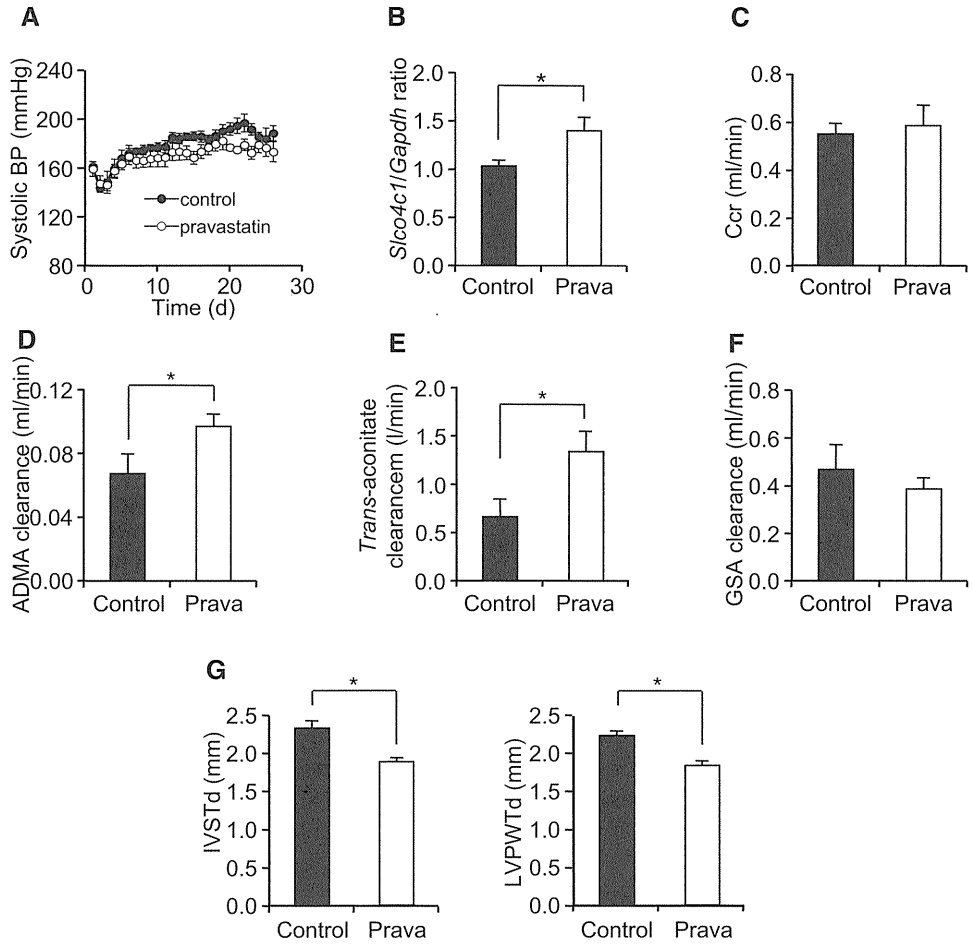

Figure 7. Effects of pravastatin *in vivo*. (A) BP in control and pravastatin-treated (0.1 mg/ml drinking water) rats after five-sixths Nx ($n = 6$ to 7 per group). (B) The mRNA expression of rat slco4c1 in the kidney after pravastatin administration ($n = 11$ per group). (C through F) Renal clearance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

moter.[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′-tccccccacttctgtttcccagtctatgt-3′ and reverse (human SLCO4C1) 5′-acgcgatctgcagaattagcttgggctc-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).

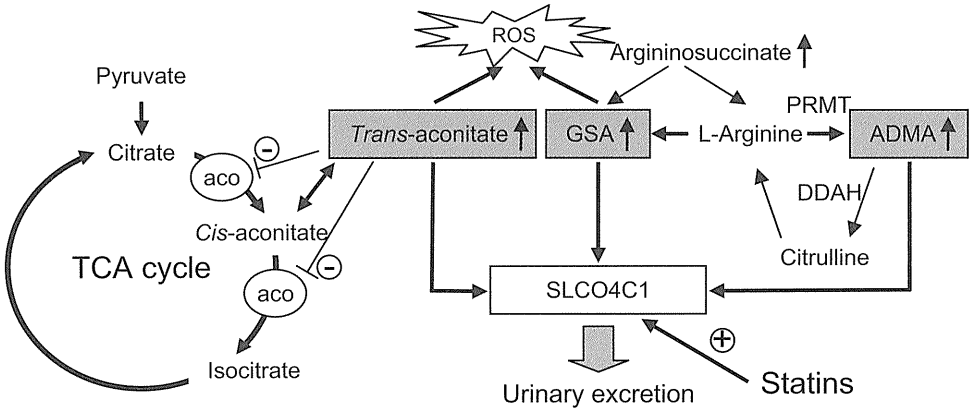

Figure 8. Uremic toxins and SLCO4C1 transporter in renal failure. ADMA is formed by protein arginine N-methyltransferase (PRMT) from arginine and degrades to citrulline by DDAH. Note that SLCO4C1 facilitates the excretion of GSA, ADMA, and *trans*-aconitate and that statins increase the expression and the function of SLCO4C1, resulting in reductions of the uremic toxins and BP. *Trans*-aconitase inhibits aconitase activity and induces reactive oxygen species (ROS). Aco, aconitase.

Nephrectomized Rat Model and BP Measurement

Five-sixths nephrectomized rats were generated as previously reported.[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 formula[42] eGFR (ml/min per 1.73 m^2) = 175 × creatinine$^{-1.154}$ × age$^{-0.203}$ × 0.742 (if female) × 0.741.

Measurement of Reactive Oxygen Species

The free radical formation within the human kidney proximal cell line HK-2 evoked by *trans*-aconitate (100 μM) was monitored by measurement of the changes in fluorescence resulting from the oxidation of dihydroethidium to ethidium as the increase of ethidium production (U/s)[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 μg 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 LTB_4) 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 crossed-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-AACCCCACCCCAA-3′ and reverse primer 5′-CTAAGTATGGT-GGAGGAAAGGGTG-3′). Nuclear and cytoplasmic fraction extracts were prepared and Western blotting was performed as described previously[39] using antibodies against AhR, Lamin B (Santa Cruz Biotechnology, Santa Cruz, CA), and α-tubulin (Sigma-Aldrich, St. Louis, MO).

Real-Time PCR Analysis

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

Statistical Analysis

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

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DISCLOSURES

None.

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

Qi Guo[a], Takefumi Mori[a,b], Yue Jiang [a], Chunyan Hu[a], Yusuke Osaki[a], Yoshimi Yoneki[a], Ying Sun[a], Takuma Hosoya[a], Akihiro Kawamata[a], Susumu Ogawa[a], Masaaki Nakayama[c], Toshio Miyata[d] and Sadayoshi Ito[a]

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) 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

[a]Division of Nephrology, Endocrinology and Vascular Medicine, Tohoku University Graduate School of Medicine, [b]Health Administration Center, Tohoku University, [c]Research Division of Dialysis and Chronic Kidney Disease and [d]Center for Translational and Advanced Research, Tohoku University Graduate School of Medicine, Sendai, Japan

Correspondence to Takefumi Mori, MD, PhD, Division of Nephrology, Endocrinology and Vascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryocho, Aoba-ku, Sendai 980-8574, Japan
Tel: +81 22 717 7163; fax: +81 22 717 7168;
e-mail: tmori2i@mail.tains.tohoku.ac.jp

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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 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

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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, Shizuoka, 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 tetraethoxyprophane 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% thichloroacetic 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

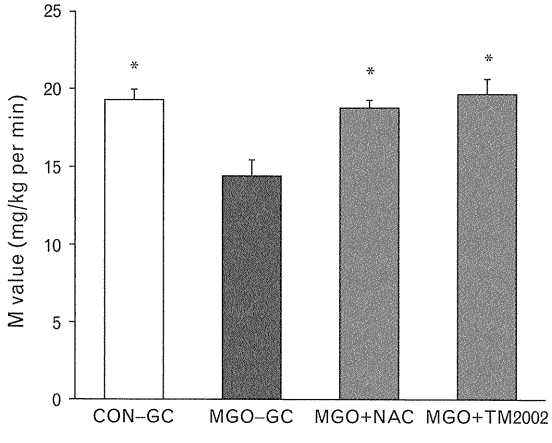
All results were expressed as the means ± SE. Intergroup comparisons of SBP over the study period were made by repeated measures ANOVA with Bonferroni/Dunn test. All other data analyses were performed by Bonferroni/Dunn test for multiple comparisons after one-way analysis of variance. Values of $P <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

Results

Insulin sensitivity

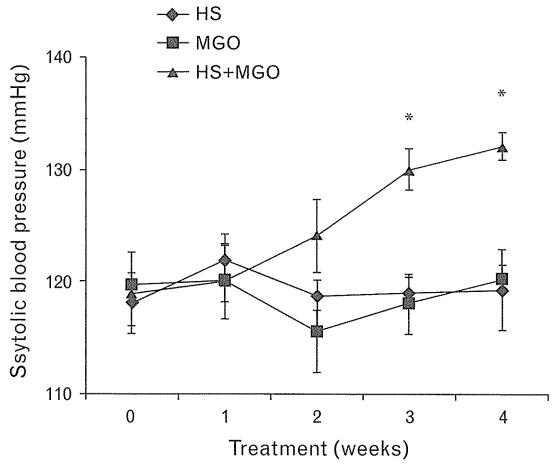
After 4 weeks of treatment, there were no significant intergroup differences in body weight (BW; CON-GC, 304 ± 5 g; MGO-GC, 312 ± 7 g; MGO + NAC, 308 ± 10 g; MGO + TM2002, 307 ± 6 g) or in fasting blood glucose (CON-GC, 6.4 ± 0.2 mmol/l; MGO-GC, 6.4 ± 0.3 mmol/l; MGO + NAC, NAC, 6.4 ± 0.2 mmol/l; MGO + 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

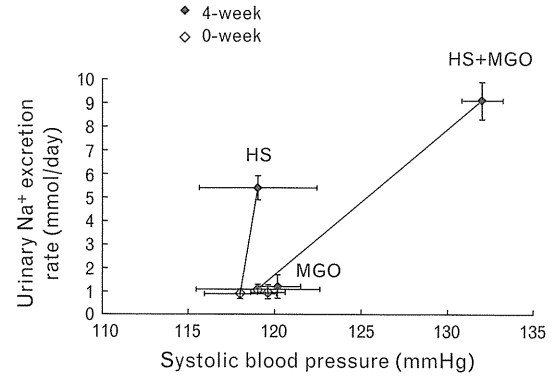


Systolic blood pressure responses to high salt diet or/and methylglyoxal (MGO) treatment at baseline and throughout the 4-week study. Data are the means ± SE. $n = 6$ for each group. *$P < 0.01$ vs. HS or MGO group. HS, high-salt diet; MGO, methylglyoxal.

Blood pressure

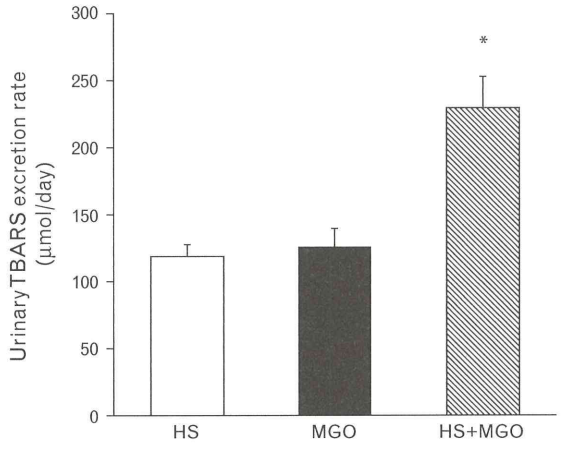
The SBPs of the rats during the 4-week experimental period are shown in Fig. 2. At week 0, there were no significant differences in SBP among the groups. Neither MGO alone nor high-salt diet alone significantly changed SBP during the 4-week experimental period (MGO, 120 ± 1 mmHg to120 ± 2 mmHg; high-salt 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 ± 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

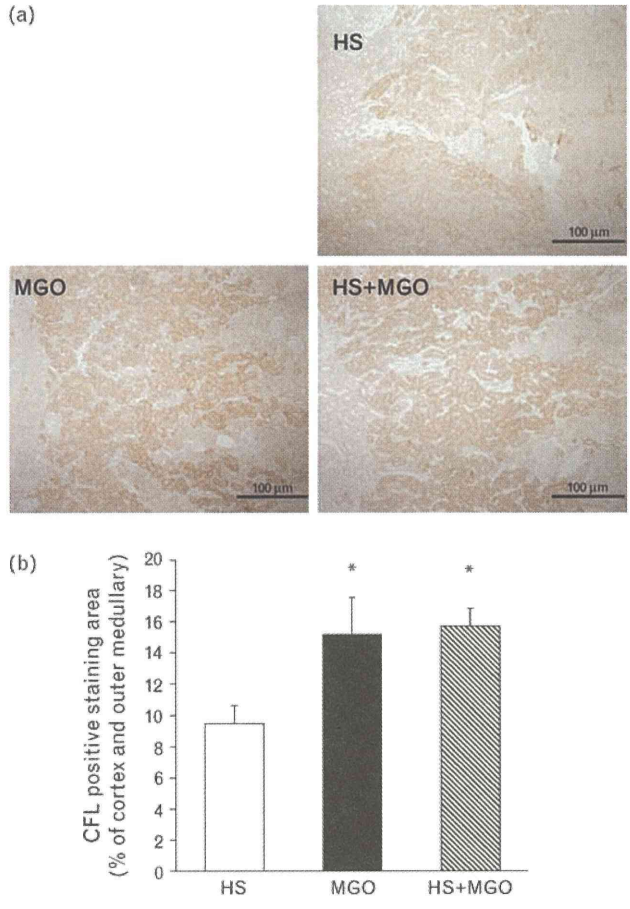
The 24-h urinary excretion of TBARS value in the high-salt diet + MGO group (229 ± 23 μmol/day) was obviously higher than that in either the MGO (125 ± 14 μmol/day) or high-salt diet (118 ± 9 μmol/day) group (Fig. 4).

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 ± 2.3%) or a combination of high-salt diet and MGO (15.7 ± 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 ± 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 ± 0.8%, $P < 0.001$) group was clearly higher than that in the high-salt diet (7.2 ± 0.8%) or MGO (6.7 ± 0.4%) group (Fig. 6).

Fig. 5



Representation of N^e-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 ± SE. $n = 6$ for each group. *$P < 0.05$ vs. HS group.

Body and urine compositions

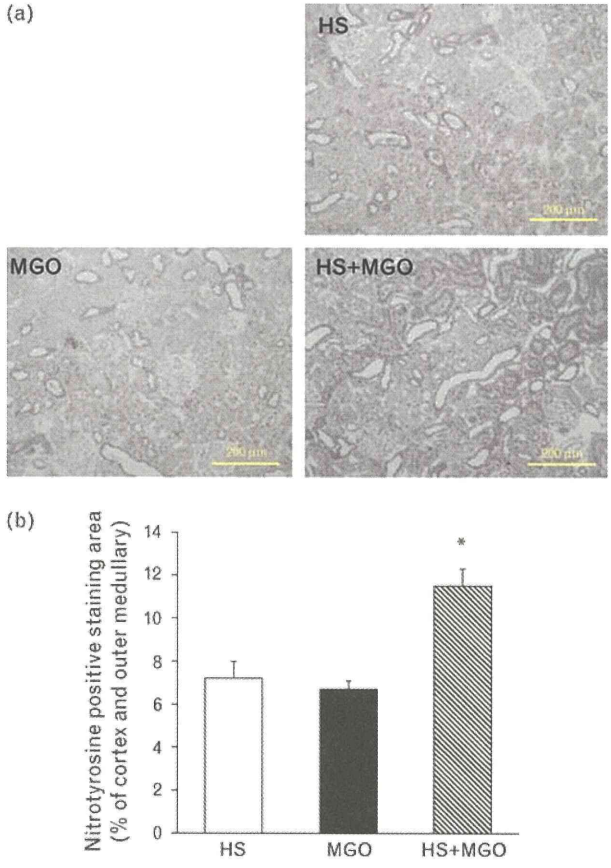
There were no significant intergroup differences in BW, urinary albumin excretion (Ualb) and Ualb/creatinine ratio (Table 1). The kidney weight (KW) was significantly greater in the high-salt diet + MGO group than in the high-salt diet or MGO group. The KW/BW ratio was significantly higher in the high-salt 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

Fig. 6



Representation of nitrotyrosine positive staining area in rats. (a) Representative images of nitrotyrosine in the kidney from Sprague–Dawley rats. (b) Percentage of nitrotyrosine positive staining area of the cortex and outer medullary 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 ± SE. $n = 6$ for each group. *$P < 0.001$ vs. HS or MGO group.

metabolite may play a role in the development of insulin resistance. Several studies have shown that MGO inhibited the insulin signaling in cultured skeletal muscle cells [16], and in adipose tissue of fructose-induced hypertensive rats [17]. However, whether increased MGO would directly contribute to the development of insulin resistance in a normotensive state is still unsettled. To the best of our knowledge, the present study is the first to investigate the effects of MGO on insulin sensitivity by using a glucose clamp in normotensive Sprague–Dawley rats. We also successfully demonstrated that MGO-induced salt sensitivity. The results of the present study indicate that high plasma levels of MGO are implicated in the pathophysiology of chronic kidney disease.

The present study demonstrated that 4-week treatment with 1% MGO in drinking water significantly increased the M value (a marker of insulin resistance) without altering the blood pressure in Sprague–Dawley rats (Figs. 1 and 2). We also found that 4-week treatment with not only NAC, an aldehyde binding compound as a MGO scavenger, but also TM2002, a novel AGE inhibitor [12], completely improved MGO-induced insulin resistance to a level of control group. As enhanced AGEs have been demonstrated to increase insulin resistance [18], it may be that MGO-induced insulin resistance was at least partly mediated through AGEs in the present study.

Although we do not have further evidence for the exact mechanism of the MGO-induced insulin resistance in the present study, there are several possible mechanisms by which MGO affects insulin resistance. First, MGO and MGO-induced AGEs can induce nonenzymatic modifications of various amino acid residues (e.g., lysine and arginine) that are generally present in the active sites of various insulin-signaling proteins, and MGO-induced structural modifications of these proteins might cause impaired insulin-signaling transduction. Jia *et al.* [17] reported that elevation of the endogenous MGO level in fructose-fed rats reduced insulin receptor substrate (IRS-1)/phosphatidylinositol-3-kinase (PI3K) association and altered PI3K activity, which may lead to the decrease in insulin-stimulated glucose uptake in adipose tissue, thereby contributing to insulin resistance. Furthermore, MGO modifies insulin by attaching to an internal arginine residue in the β-chain of insulin [19]. The formation of this MGO-insulin adduct decreases insulin-mediated glucose uptake, impairs autocrine control of the insulin secretion, and decreases insulin clearance. These structural and functional abnormalities of insulin molecule may contribute to the pathogenesis of insulin resistance.

Role of methylglyoxal in the development of hypertension

There have been several reports implicating MGO in the development of hypertension, but the exact mechanism is not yet fully understood. Vasdev *et al.* [20] have

Table 1 Body weight, kidney weight, kidney weight/body weight, urinary albumin excretion (Ualb) and Ualb/creatinine ratio (Ualb/Cre) after the 4-week study

	BW (g)	KW (mg)	KW/BW (mg/g)	Ualb (μg/day)	Ualb/Cre (μg/mg)
HS	302 ± 4	1000 ± 17	3.3 ± 0.1	429 ± 96	40 ± 9
MGO	290 ± 12	994 ± 36	3.5 ± 0.2	210 ± 25	21 ± 1
HS + MGO	296 ± 10	1107 ± 41*,†	3.8 ± 0.1*	570 ± 278	50 ± 28

Data are the means ± SE. $n = 6$ for each group. BW, body weight; HS, high-salt-fed rats; MGO, normal-fed rats treated with methylglyoxal; HS + MGO, high-salt-fed rats treated with methylglyoxal; KW, kidney weight; Ualb, urinary albumin excretion; Ualb/Cre, Ualb/creatinine ratio. *$P < 0.05$ vs. HS group. †$P < 0.05$ vs. MGO group.

proposed a role of MGO in the Ca^{2+} channels of the arteries. They have demonstrated that methylglyoxal binds to the sulfhydryl groups of vascular Ca^{2+} channels and increases intracellular cytosolic Ca^{2+} levels, which enhance vascular tension. Oxidative stress is another mechanism that could explain MGO-induced hypertension. Wang *et al.* [2,3] have demonstrated that the MGO level was elevated in parallel with increased oxidative stress and AGEs in SHR compared with normotensive WKY rats. These results indicated that elevated MGO levels can lead to increased production of ROS and AGEs, and thereby might contribute to the development of hypertension. Enhanced production of ROS leads to structural and functional alterations, such as endothelial dysfunction and, vascular smooth muscle hypertrophy and hyperplasia, all of which contribute to the development of hypertension [21]. A number of studies [22–24] have shown that there is a synergistic relationship between MGO, AGEs and oxidative stress. Accumulation of AGEs further magnifies ROS damage by inducing glycation of the enzymes involved in the antioxidant system and by providing precursors of oxidative stress.

Vasdev *et al.* [20] have demonstrated that treatment with MGO (0.2–0.8% in drinking water) significantly increased blood pressure in WKY rats. In the present study, although we expected that MGO would induce a significant increase in blood pressure, treatment with 1% MGO alone had no effect on blood pressure for up to 4 weeks in Sprague–Dawley rats. The reason for the different results of MGO on blood pressure between in Sprague–Dawley rats and in WKY rats is not clear, but except difference in the rat strains, higher plasma angiotensin II level [25] and the oxidative stress level of renal proximal tubules [26] in WKY than Sprague–Dawley rats may be involved. In addition, although it is reported that treatment of Sprague–Dawley rats with 4% high-salt diet for 4-week increases urinary excretion rate of H_2O_2 [11], 4% high-salt diet do not increase blood pressure. In the present study, the levels of urinary TBARS excretion and nitrotyrosine expression in the kidney (markers of oxidative stress) were found to be similar between the MGO and high-salt diet groups (Figs. 4 and 6). Taken together, these results indicate that neither the increase in oxidative stress by salt loading nor the formation of AGEs by MGO loading is sufficient in itself to induce the development of hypertension, but when the two are combined, hypertension develops.

Methylglyoxal induces salt sensitivity in Sprague–Dawley rats

Interestingly, coadministration of MGO and high-salt diet significantly increased SBP and the urinary Na^+ excretion rate after 4-week treatment compared with either MGO or high-salt diet alone in the present study (Figs. 2 and 3). These data suggested that MGO caused an increase in salt sensitivity in normotensive Sprague–Dawley rats. Although the exact mechanisms of the MGO-induced salt sensitivity in normotensive Sprague–Dawley rats remains to be investigated, increased oxidative stress could be a trigger for salt sensitivity [27].

In the present study, renal nitrotyrosine expression was observed strongly in the tubules of the outer medulla. It has been shown that enhanced medullary oxidative stress plays an important role in the pathophysiology of salt-sensitive hypertension. The balance between nitric oxide and superoxide in this region, which participates in the regulation of renal medullary blood flow, has been shown to determine the level of blood pressure and salt sensitivity [28,29]. Taylor *et al.* [30] have demonstrated that renal medullary H_2O_2 concentration is higher in Dahl salt-sensitive rats than in salt-resistant ($SS13^{BN}$) control rats, determined the role of oxidative stress to the salt sensitivity. In their study, the reduction of renal medullary oxidative stress induced by the antioxidant apocynin reduced the salt sensitivity in Dahl salt-sensitive rats [30]. On the contrary, renal medullary interstitial infusion of H_2O_2 in $SS13^{BN}$ rats induced a salt-sensitive form of hypertension [31]. Increased salt sensitivity was shown to be associated with reduced medullary blood flow [31]. Therefore, the renal medullary oxidative stress observed in the present study may have reduced medullary blood flow and induced salt sensitivity.

Oral methylglyoxal intake induces renal oxidative stress and advanced glycation endproducts

The present study demonstrated that orally administered MGO could contribute to salt sensitivity and insulin resistance. Although we have not determined whether oral administration of MGO could directly increase renal MGO, the expression of CEL and nitrotyrosine in the renal tubules indicates that oral MGO has at least some effect on AGEs and oxidative stress. This is consistent with the previous report by Vasdev *et al.* [20] that oral administration of MGO increased renal aldehyde in WKY rats. In addition, it is well recognized that the rats orally given fructose developed the pathogenesis of metabolic syndrome (including hypertension and insulin resistance) and increased accumulation of endogenous MGO (plasma and tissues MGO levels) [17,32]. Therefore, oral administration of MGO comparable to that of endogenously produced MGO could be involved in the pathogenesis of renal injury, insulin resistance and hypertension. MGO and associated AGEs are also formed by nonenzymatic reactions and could be formed in heat-treated foods [33–35]. A high AGE diet has been shown to increase plasma CEL and MGO derivatives in healthy human individuals [34]. Moreover, macrovascular and microvascular function is attenuated in diabetes patients with high AGE intake, indicating that oral MGO and/or AGEs play a role in the pathogenesis of diabetic vasculopathy [33]. Therefore, we suspect that MGO also plays a role in the insulin resistance and salt sensitivity in