

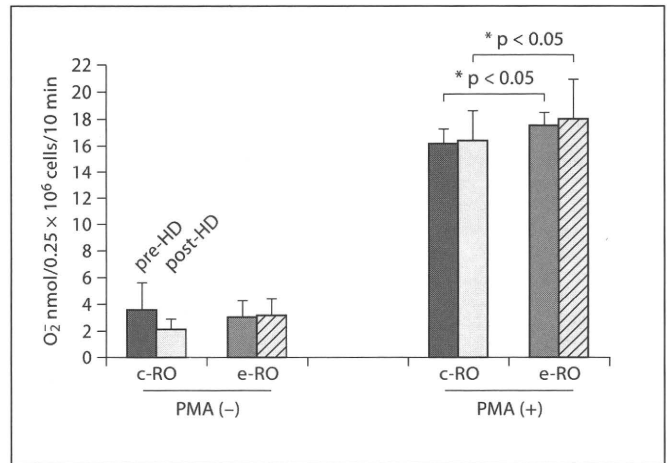
**Fig. 3.** Effect of e-RO: human neutrophil viability against MGO and/or PMA. Neutrophils from 7 healthy volunteers were suspended in PBS (n = 7). c-RO = treatment by control RO water; e-RO = treatment by RO water by electrolysis. Data are expressed as means  $\pm$  SD. \* p < 0.05.

The present study was approved by the ethical committees of Kashima Hospital and Tohoku University (No. 2006-101, No. 2007-207), and informed consent was obtained from all patients. All values are expressed as mean  $\pm$  SD. Paired t test and 1-way repeated measure analysis of variance were used for statistical analysis. p < 0.05 was considered statistically significant.

## Results

Changes in ATP-chemiluminescence of PMNs against MGO in the presence or absence of PMA are shown in figure 3. At basal levels (MGO 0  $\mu$ M) in the absence of PMA, ATP-chemiluminescence was significantly higher with e-RO than c-RO (p < 0.05). Significant decreases in ATP-chemiluminescence were associated with MGO levels in both the e-RO and c-RO groups (p < 0.001 for both groups). However, no differences were found between the e-RO and c-RO groups in MGO levels. For PMA load, further significant decreases in ATP-chemiluminescence were associated with MGO levels in the e-RO and c-RO groups (at MGO levels 0–100  $\mu$ M, p < 0.01 in both groups); however, no differences were found between c-RO and e-RO.

Figure 4 shows generated superoxide anions in both groups. Under basal levels, no differences were found between groups, whereas in stimulated conditions, levels of generated radicals were significantly higher for e-RO than for c-RO (p < 0.05 in pre-HD and post-HD).



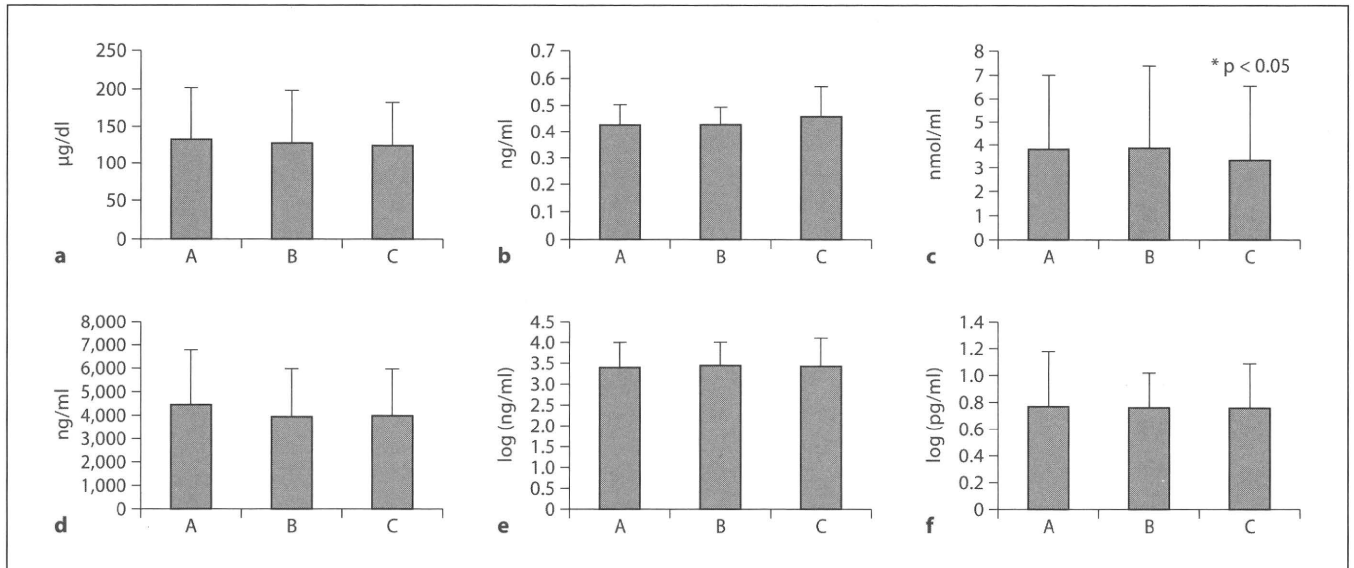
**Fig. 4.** Effect of e-RO: superoxide anion generation from human neutrophils by PMA. Neutrophils from patients on hemodialysis were suspended in PBS (n = 6). c-RO = treatment by control RO water; e-RO = treatment by RO water by electrolysis. Data are expressed as means  $\pm$  SD. \* p < 0.05.

**Table 2.** Change in laboratory parameters

	Pre	Post	p value
WBC, n/ $\mu$ l	6,795 $\pm$ 863	6,644 $\pm$ 945	0.35
Hb, g/dl	11.3 $\pm$ 1.2	11.2 $\pm$ 1.2	0.19
Creatinine, mg/dl	11.0 $\pm$ 3.2	10.6 $\pm$ 3.0	0.07
BUN, mg/dl	64.6 $\pm$ 6.2	60.8 $\pm$ 9.0	0.1

Data are means  $\pm$  SD. Blood samples were obtained from the arteriovenous fistula before starting dialysis. Pre = e-HD day 1 (fig. 1); Post = c-HD day 13 (fig. 1); WBC = white blood cells; Hb = hemoglobin; BUN = blood urea nitrogen.

Changes in laboratory parameters during the clinical trial of e-HD are shown in table 2. No changes were observed in white blood cell count, hemoglobin, blood urea nitrogen and creatinine levels. Figure 5 shows changes in inflammatory or oxidative stress markers in the blood. There were significant decreases in plasma methylguanidine levels (p < 0.05; fig. 5c). There were also significant decreases in mean blood pressure during the study in the c-HD and e-HD groups between the pre- and post-HD sessions (p < 0.01 and p < 0.001, respectively; table 3). No adverse symptoms were observed in any patient.



**Fig. 5.** Changes in laboratory variables after HD using the e-RO based HD solution. **a** Creatol. **b** 8-OHdG. **c** Methylguanidine. **d** Pentosidine. **e** Highly sensitive C reactive protein. **f** Interleukin-6. Data are expressed as means  $\pm$  SD. A = the start of the first e-HD session (first week); B = the start of the fourth e-HD session (second week); C = the start of c-HD after 12 e-HD sessions (post-e-HD). \*  $p < 0.05$ .

## Discussion

It has been demonstrated that e-RO exhibits antioxidative capacity [13–15]. The present study aimed to investigate the biological effect of e-RO on the viability of PMNs, and the clinical feasibility of applying this technology to HD treatment.

Plasma MGO levels are reportedly elevated in patients on dialysis [19, 20]. MGO is highly reactive and biologically toxic due to the formation of advanced glycation end products, protein and DNA modifications [21–23]. Recently, we demonstrated that MGO plays a role in the enhancement of injury to PMNs in combination with  $H_2O_2$  [24], which may be involved with the pathological mechanism of microinflammation in dialysis patients.

In the c-RO group in the present study (fig. 3), decreased viabilities of PMNs were found to be associated with the MGO level, and the toxicity of MGO was further increased in the presence of PMA. Basal cellular viability was better preserved in the e-RO group than in c-RO group. Furthermore, MGO-related PMN injury in the e-RO group was relatively ameliorated compared with the c-RO group, although differences did not reach statistical significance. In addition, in PMNs of uremic patients (fig. 4), the cellular function to generate superoxide anions by a physiological respiratory burst was significantly higher with e-RO than with c-RO.

**Table 3.** Blood pressure and heart rate pre- and post-dialysis

	c-HD (n = 12)	e-HD (n = 12)	p value
Pre-dialysis			
SBP, mm Hg	165 $\pm$ 25	157 $\pm$ 21	<0.01
DBP, mm Hg	85 $\pm$ 12	81 $\pm$ 10	<0.05
MBP, mm Hg	112 $\pm$ 15	106 $\pm$ 12	<0.01
HR, beats/min	76 $\pm$ 12	78 $\pm$ 14	0.71
BW gain, %	3.8 $\pm$ 0.85	4.0 $\pm$ 0.93	0.9
Post-dialysis			
SBP, mm Hg	124 $\pm$ 19	117 $\pm$ 15	<0.01
DBP, mm Hg	69 $\pm$ 11	64 $\pm$ 9	<0.001
MBP, mm Hg	87 $\pm$ 13	81 $\pm$ 11	<0.001
HR, beats/min	80 $\pm$ 12	81 $\pm$ 14	0.63
Volume removed, kg	2.2 $\pm$ 0.8	2.4 $\pm$ 0.8	0.97

Data are the mean  $\pm$  SD levels of 12 HD sessions in each patient. BW gain = Inter-dialytic body weight gain over dry weight; c-HD = hemodialysis using control reverse osmosis water; DBP = diastolic blood pressure; e-HD = hemodialysis using reverse osmosis water by electrolyzed water; HR = heart rate; MBP = mean blood pressure; pre-dialysis = just before the hemodialysis session; post-dialysis = just after the hemodialysis session; SBP = systolic blood pressure; volume removed = intra-dialytic volume removal per HD session.

These findings indicate that the application of e-RO does not stimulate PMNs, but rather benefits PMNs in preserving cellular viability as compared with c-RO. However, suppression of MGO toxicity by e-RO application was not clearly demonstrated in this study (fig. 3). In terms of the lack of e-RO effect on MGO cytotoxicity in this study (fig. 3), we think that the change in chemical properties of e-RO, such as levels of dissolved hydrogen, may contribute to our findings, as indicated by recent studies that suggested a significant role for dissolved hydrogen in ameliorating oxidative stress [25, 26]. Further studies are needed in this area to address the context of the preservation of e-RO chemical properties.

Next, we examined the feasibility of the application of this technology to regular HD treatment. No symptomatic adverse events were observed during the study period, although there were nonsymptomatic reductions in mean blood pressure before and after the HD session. No changes were found in laboratory parameters or in interdialytic changes of body weight, which may indicate the feasibility of an e-HD system.

In previous reports, Huang et al. [13, 14] showed decreases in surrogate markers of inflammation and oxidative stress following the introduction of an e-HD system. In the present short trial, plasma methylguanidine was significantly decreased, but no other parameters, including C-reactive protein, interleukin-6, creatol and 8-OHdG, were changed (fig. 5). We propose the following

reasons for these contradictory results. First, methylguanidine is the nonspecific oxidative end product that could reflect the overall oxidative milieu, and thus could be changed significantly. Second, we only enrolled clinically stable patients, and thus those who presented with inflammation or malnutrition were not included in the study. This clinical background might influence the results of our study.

Interestingly, MBP was decreased by the introduction of e-HD. Taken together, the findings of decreased MBP and no changes in heart rate or inter-dialytic body weight gain lead us to speculate that e-HD may lead to vasodilation. Vasoconstrictive action by radical oxygen species such as peroxynitrite could play a role in increased blood pressure in HD patients, and dissolved hydrogen in EW may reduce superoxide anions, as previously reported [8], during the course of e-HD to suppress its pathological process. Further studies are needed in this area.

There are some unresolved issues in the present HD system. Data on the microbiological quality of the dialysis solution were not available, and water volume consumed per session was not clearly measured. These issues need to be clarified in follow-up studies.

In conclusion, the present study demonstrated the capacity of e-RO to preserve the viability of PMNs, and the clinical feasibility of using this water for HD treatment. The clinical application of this technology may improve bio-compatibility of HD treatment.

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

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

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

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

### Introduction

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

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

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

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

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

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

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

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

## Methods

### Animals

Eight-week-old male Sprague–Dawley rats (SLC, 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  $\text{Na}^+$ , albumin and creatinine were measured by a standard autoanalysis technique (Synchro-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 tetrathoxypropane 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%, 0.25N HCl and 15% trichloroacetic acid) for 15 min in boiling water, then cooled and centrifuged at room temperature for 10 min at 3000g. 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- $\mu\text{m}$ . Sections were immunostained with CEL as a marker of AGEs and carbonyl stress, and with nitrotyrosine as a marker of peroxynitrite formation [15]. For CEL and nitrotyrosine immunostaining, deparaffinized sections were retrieved by microwaving in 10 mmol/l citrate buffer (pH 7.0) for 5 min, staining with anti-CEL (KH-025, diluted 1:50; Trans Genic, Kumamoto, Japan) or antinitrotyrosine (AB5411, diluted 1:200; Chemicon, Temecula,

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

**Data analysis**

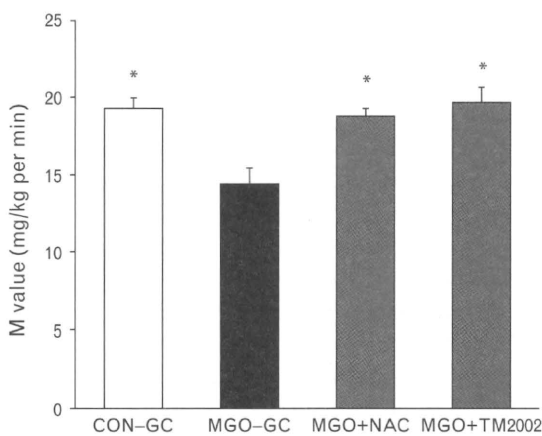
All results were expressed as the means ± SE. Intergroup comparisons of SBP over the study period were made by repeated measures ANOVA with Bonferroni/Dunn test. All other data analyses were performed by Bonferroni/Dunn test for multiple comparisons after one-way analysis of variance. Values of *P* 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

**Results**

**Insulin sensitivity**

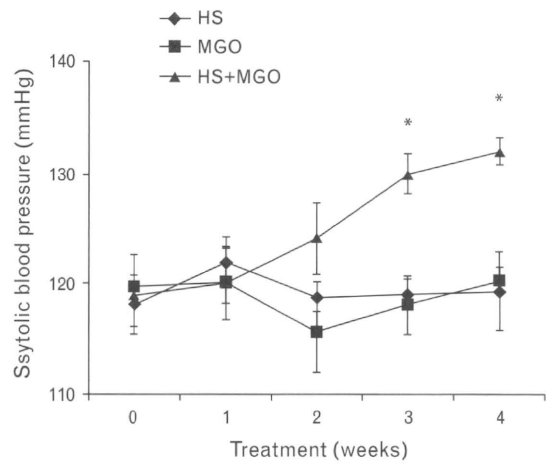
After 4 weeks of treatment, there were no significant intergroup differences in body weight (BW; CON-GC, 304 ± 5 g; MGO-GC, 312 ± 7 g; MGO + NAC, 308 ± 10 g; MGO + TM2002, 307 ± 6 g) or in fasting blood glucose (CON-GC, 6.4 ± 0.2 mmol/l; MGO-GC, 6.4 ± 0.3 mmol/l; MGO + NAC, 6.4 ± 0.2 mmol/l; MGO + TM2002,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

**Fig. 1**



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

**Fig. 2**

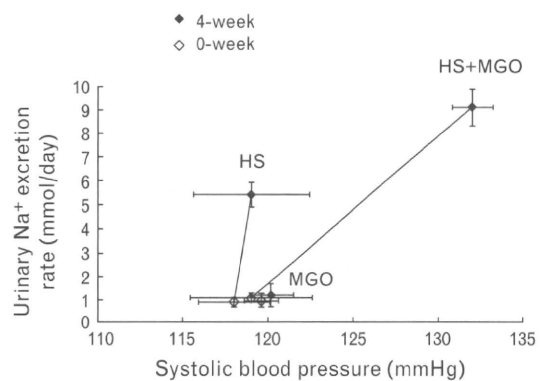


Systolic blood pressure responses to high salt diet or/and methylglyoxal (MGO) treatment at baseline and throughout the 4-week study. Data are the means ± SE. *n* = 6 for each group. \**P* < 0.01 vs. HS or MGO group. HS, high-salt diet; MGO, methylglyoxal.

**Blood pressure**

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

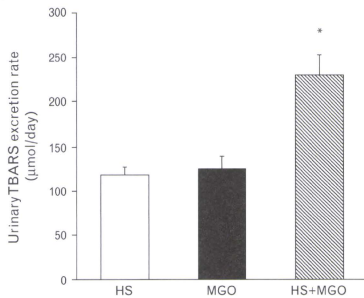
**Fig. 3**



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



Fig. 4



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

#### Twenty-four-hour urinary excretion of $\text{Na}^+$

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

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

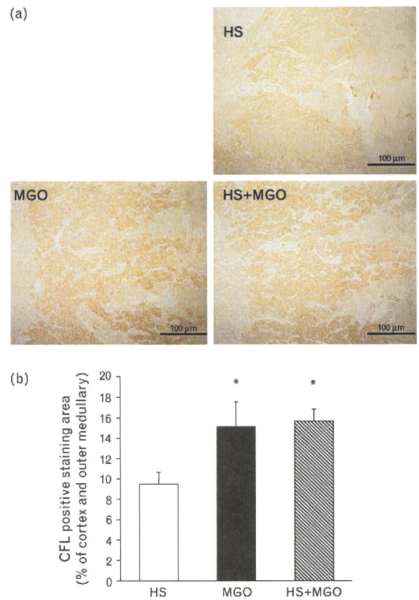
The 24-h urinary excretion of TBARS value in the high-salt diet + MGO group ( $229 \pm 23 \mu\text{mol/day}$ ) was obviously higher than that in either the MGO ( $125 \pm 14 \mu\text{mol/day}$ ) or high-salt diet ( $118 \pm 9 \mu\text{mol/day}$ ) group (Fig. 4).

#### Immunohistochemistry of the kidney

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

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

Fig. 5



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

#### Body and urine compositions

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

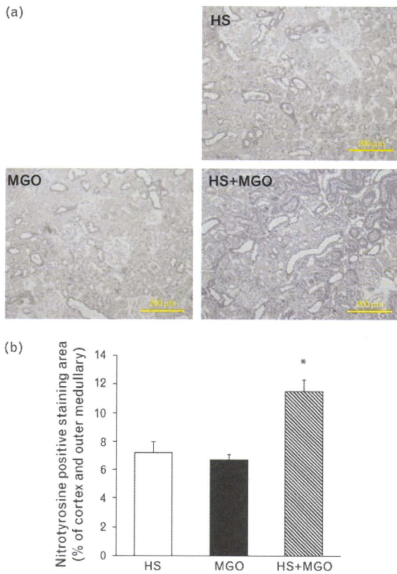
#### Discussion

##### Methylglyoxal increases insulin resistance in Sprague-Dawley rats

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



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  $\pm$  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  $\beta$ -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 ( $\mu$ g/day)	Ualb/Cre ( $\mu$ g/mg)
HS	302 $\pm$ 4	1000 $\pm$ 17	3.3 $\pm$ 0.1	429 $\pm$ 96	40 $\pm$ 9
MGO	290 $\pm$ 12	994 $\pm$ 36	3.5 $\pm$ 0.2	210 $\pm$ 25	21 $\pm$ 1
HS + MGO	296 $\pm$ 10	1107 $\pm$ 41 <sup>*†</sup>	3.8 $\pm$ 0.1*	570 $\pm$ 278	50 $\pm$ 28

Data are the means  $\pm$  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. <sup>†</sup> $P < 0.05$  vs. MGO group.

proposed a role of MGO in the  $\text{Ca}^{2+}$  channels of the arteries. They have demonstrated that methylglyoxal binds to the sulfhydryl groups of vascular  $\text{Ca}^{2+}$  channels and increases intracellular cytosolic  $\text{Ca}^{2+}$  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  $\text{H}_2\text{O}_2$  [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  $\text{Na}^+$  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  $\text{H}_2\text{O}_2$  concentration is higher in Dahl salt-sensitive rats than in salt-resistant (SS13<sup>BN</sup>) 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  $\text{H}_2\text{O}_2$  in SS13<sup>BN</sup> 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

diabetes, and this hypothesis should be tested in a future clinical study.

### Study limitation

There was a notable limitation in the present study. Although the limited sensitivity in the detection of insulin resistance, we tried to look at the maximum response and used the highest concentration of MGO as possible. In the present study, 4-week treatment with 1% MGO in drinking water increased plasma MGO level by about 7.5-fold compared with the control rats in Sprague–Dawley rats (data not shown). The increased magnification of plasma MGO level in the present study is as high as that in patients with chronic kidney disease (CKD) stages 5 compared with the healthy controls (about 5–10 fold) [36]. With the technical limitation of measurements and the control of plasma and/or tissue concentration of MGO, we were not able to determine whether MGO on insulin resistance or salt sensitivity were dose dependent. Further investigation is required in the future study by amelioration of these techniques.

In summary, we have demonstrated that MGO induces insulin resistance as well as salt sensitivity of blood pressure in normotensive Sprague–Dawley rats. Our results suggest that these effects of MGO may be mediated at least in part by increased oxidative stress or AGEs formation, or both. Our present study provides further evidence that MGO is one of the causative factors in the pathogenesis of insulin resistance and salt-sensitive hypertension. Antioxidants and ACE inhibitors may be useful for the treatment of chronic kidney disease individuals with insulin resistance and salt sensitivity.

### Acknowledgement

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Letter

## Carbonated soft drinks and carbonyl stress burden

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

**Key words:** Carbonated soft drink, MG, Carbonyl stress

### INTRODUCTION

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

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

stress burden is of clinical importance.

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

### MATERIALS AND METHODS

#### Beverage samples

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

#### Subjects

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

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

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

<sup>a</sup>MG, methylglyoxal

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

### Laboratory analyses

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

### Statistical analyses

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

## RESULTS AND DISCUSSION

The MG concentrations in 4 types of carbonated soft

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

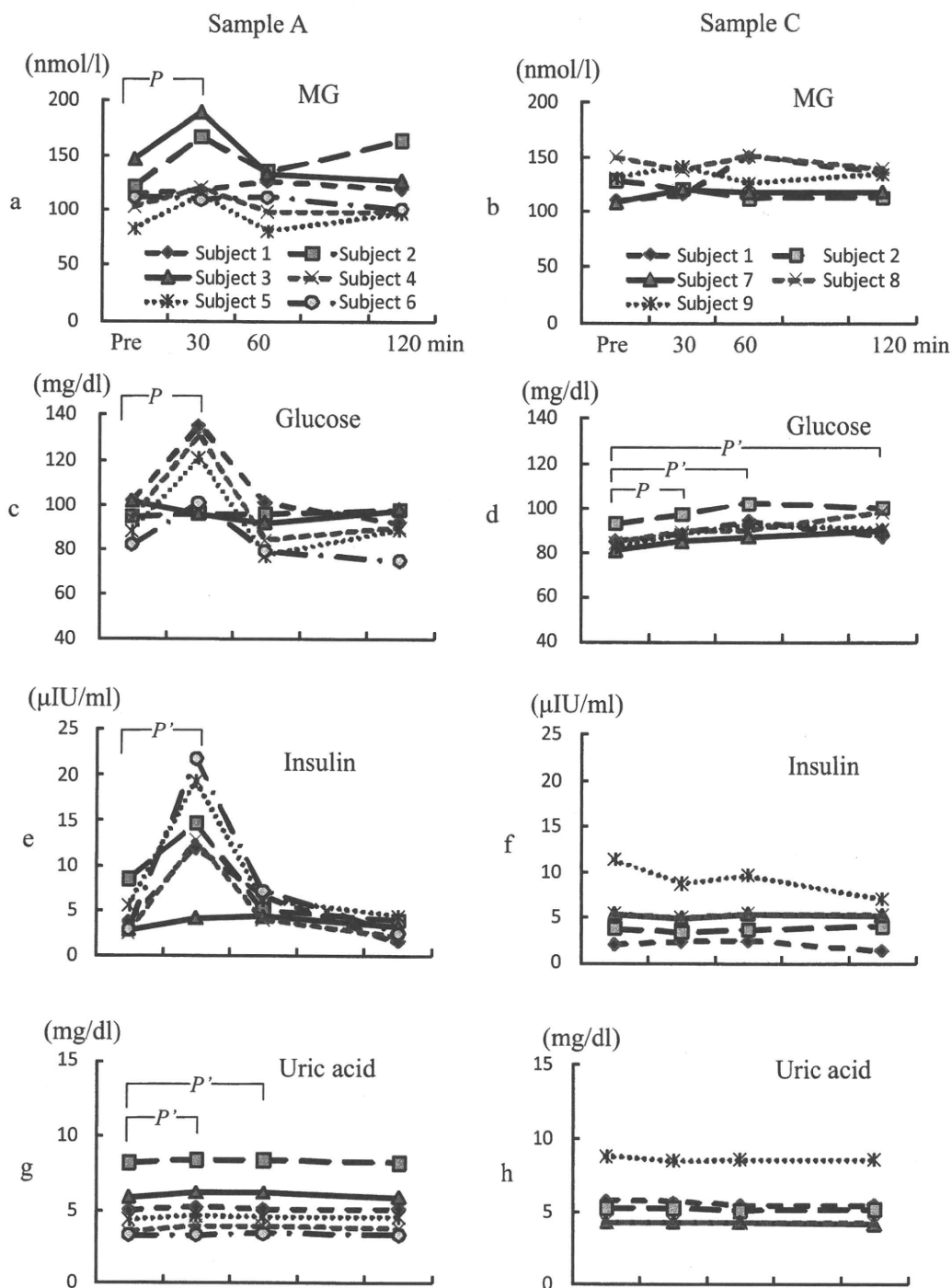
We examined the changes in plasma MG levels after intake of sample A, which contained high glucose levels and the highest level of MG among the 4 drinks, and after intake of sample C, a diet-type drink. As shown in Fig. 1, plasma MG, glucose and insulin were increased in most subjects at 30 min after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

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

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

In conclusion, glucose-containing carbonated soft

## Carbonated soft drinks and carbonyl stress



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



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

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## 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.

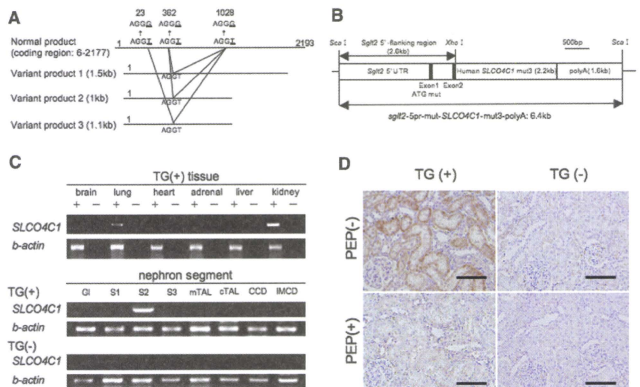
## RESULTS

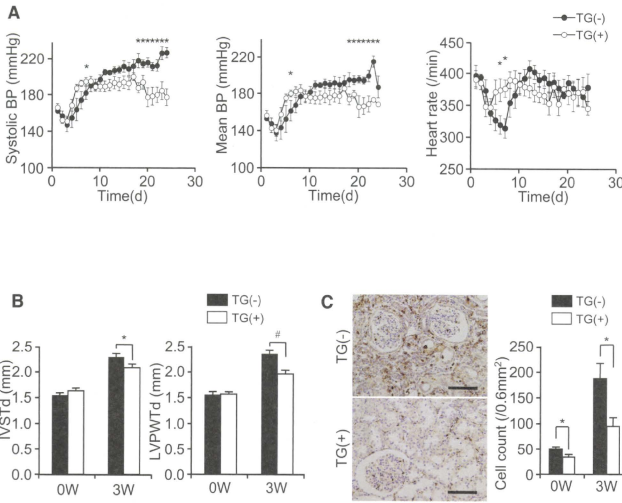
### Generation of TG Rat Harboring Human SLCO4C1 in the Kidney

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-acceptor 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.

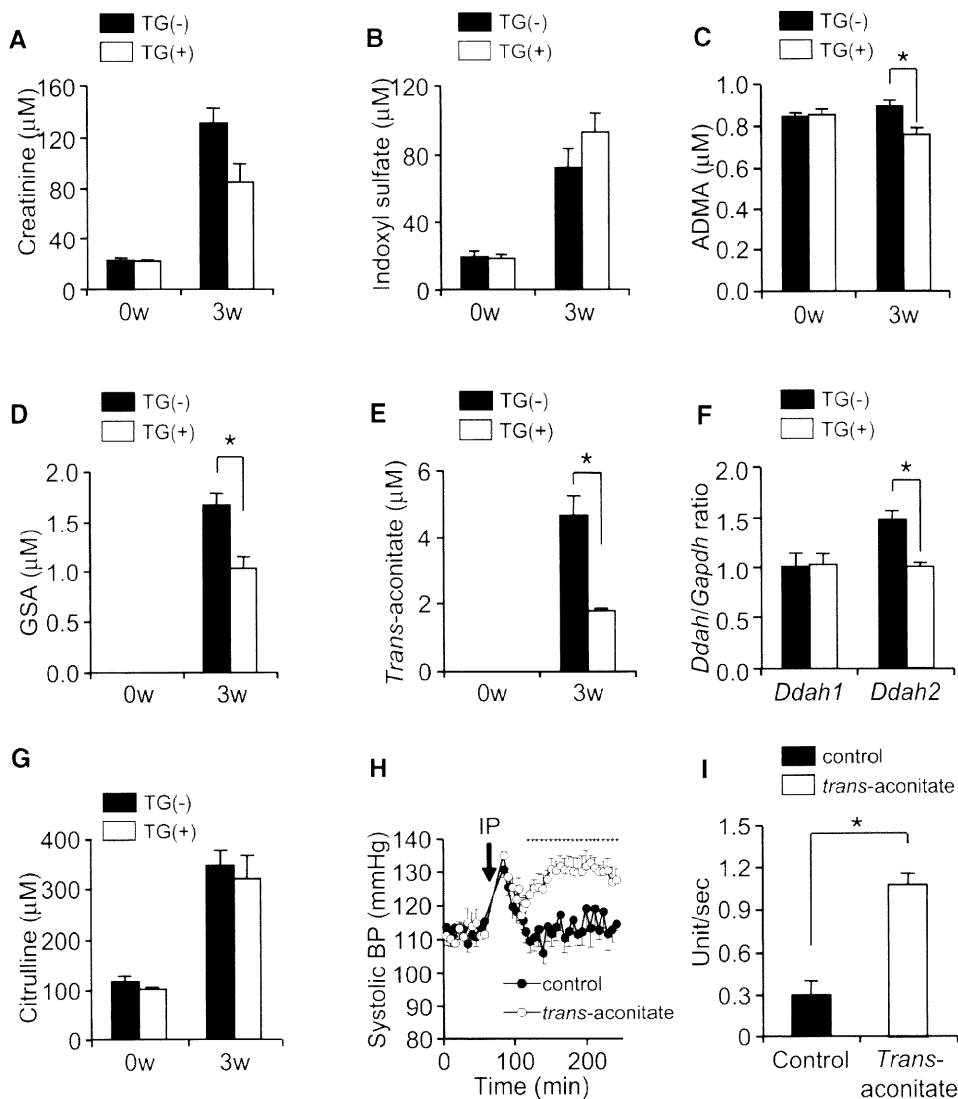
#### Elimination of Uremic Toxins in TG(+ ) Rats

To understand the mechanism by which SLCO4C1 exerted antihypertensive 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 no difference in the expression levels of other rat transporters (*slo4c1*, *oatp1*, *oatp3*, *oatp5*, *abc11*, *mrp2*, *mdr1*, and *mcl1*).

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 (GGCACGCCACGCCG). 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>