

*Full Paper***Urinary Angiotensinogen as a Novel Early Biomarker of Intrarenal Renin–Angiotensin System Activation in Experimental Type 1 Diabetes**Masumi Kamiyama^{1,2}, Andrea Zsombok^{1,2,3}, and Hiroyuki Kobori^{1,2,3,*}*Departments of ¹Physiology and ³Medicine, ²Hypertension and Renal Center of Excellence, Tulane University Health Sciences Center, New Orleans, LA 70112, USA**Received March 28, 2012; Accepted May 8, 2012*

Abstract. Urinary excretion of albumin (UAlb) is used clinically as a marker of diabetic nephropathy (DN). Although DN was thought to be a unidirectional process, recent studies demonstrated that a large proportion of patients diagnosed with DN reverted to normoalbuminuria. Moreover, despite the normoalbuminuria, one-third of them exhibited reduced renal function even during the microalbuminuric stage. This study was performed to investigate whether urinary angiotensinogen (UAGT) level may serve as a useful marker of the early stage of experimental type 1 diabetes (T1DM). T1DM was induced by a single intraperitoneal injection of streptozotocin. Control mice were injected with citrate buffer. Two days after streptozotocin injection, half of the mice received continuous insulin treatment. Our data showed that UAlb excretion was increased 6 days after streptozotocin injection compared to controls, whereas UAGT excretion was increased at an earlier time point. These increases were reversed by insulin treatment. The UAGT to UAlb ratio was increased in diabetic mice compared to control mice. Furthermore, the increased AGT expression in the kidneys was observed in diabetic mice. These data suggest that UAGT might be useful as a novel early biomarker of activation of the renin–angiotensin system in experimental type 1 diabetes.

Keywords: streptozotocin-induced type 1 diabetic mice, angiotensinogen, biomarker, renin–angiotensin system, diabetic nephropathy

Introduction

Microalbuminuria is the most commonly used early clinical indicator of diabetic nephropathy (DN) in both type 1 and type 2 diabetic patients (1, 2). Previously, DN was thought to be a unidirectional process that starts with microalbuminuria and leads to end-stage renal failure (3). Therefore, the presence of albumin (Alb) in the urine has been considered predictive of the subsequent development and clinical progression of DN. However, it was recently shown that a large proportion of type 1 diabetic patients with DN revert to normoalbuminuria, of which one-third of them exhibited reduced renal function even during the microalbuminuric stage (4). Therefore, a more sensitive and specific marker for DN, rather than urinary Alb (UAlb) excretion, is needed. The mechanisms

of DN pathogenesis are multifactorial (5, 6). In 1985, a clinical trial indicated that the activation of the intrarenal renin–angiotensin system (RAS) had a potential role in the mechanism of DN (7). Since then, it has been reported that angiotensin-converting enzyme (ACE) inhibitors, angiotensin II-receptor blockers (ARBs), or a dual blockade of the RAS (ACE inhibitors and ARBs) provide renoprotection in patients with type 1 (8, 9) or type 2 (10–14) diabetes, and some reports show the result is independent of systemic blood pressure changes (10, 13).

Others and we have previously reported that the intrarenal RAS plays an important role in the progression of DN (15–20). Especially, angiotensinogen (AGT) expression in the kidneys is consistently increased in the diabetic condition. Moreover, urinary AGT (UAGT) level was enhanced in Zucker diabetic fatty type 2 diabetic rats (21) and db/db type 2 diabetic mice (22). Recent evidence suggests that AGT is constitutively released not only in the liver but also in the proximal tubules of the

*Corresponding author. hkobori@tulane.edu
Published online in J-STAGE on July 31, 2012 (in advance)
doi: 10.1254/jphs.12076FP

kidney (6). AGT is the only known substrate for renin, which is the rate-limiting enzyme of the RAS (23, 24). Because the concentration of AGT is close to the Michaelis-Menten constant for renin, not only renin levels but also AGT levels are thought to affect the formation rate of angiotensin peptides (25, 26).

Recently, clinical studies showed that UAGT level could be a potential marker of the intrarenal RAS status in diabetes (27, 28). In hypertensive DN patients who received ARBs, urinary levels of AGT, Alb, inflammatory markers, and oxidative stress, as well as blood pressure were decreased. This suggests that the renoprotective effect of ARBs may involve suppression of intrarenal AGT levels in the patients with type 2 diabetes (27). Furthermore, an increase in UAGT levels was higher in normoalbuminuric patients with type 1 diabetes compared to control subjects, even though increased AGT was not observed in plasma (28). These observations suggest that UAGT may function as a candidate early marker for intrarenal RAS activation. To confirm our hypothesis, we investigated UAGT levels using a streptozotocin (STZ)-induced type 1 diabetic mouse model with clear onset of diabetes. This model does not have complications related to obesity and hyperlipidemia that are also associated with RAS activation; therefore, we can investigate the role of AGT after the induction of hyperglycemia, demonstrating the importance of UAGT level in patients with type 1 diabetes.

In this study, we tested the hypothesis that increased UAGT excretion is present prior to the onset of UAlb in STZ-induced type 1 diabetic mice, and UAGT might be useful as an early biomarker of activation of the RAS in experimental type 1 diabetes.

Materials and Methods

Research design

All procedures and protocols used in this study were approved by the Institutional Animal Care and Use Committee of the Tulane University Health Sciences Center. Animals used in this study were male Hsd:ICR mice (CD-1[®]; Harlan Laboratories, Indianapolis, IN, USA). Mice were maintained in 12-h light/dark cycles with access to food and water ad libitum (29). At 6 weeks of age, CD-1 mice received a single intraperitoneal injection of 200 mg/kg STZ (Fisher Health Care, Houston, TX, USA) dissolved in 0.05 M citrate buffer, pH 4.5 (n = 10). Citrate buffer alone was used in control mice (n = 10, day 0) (29, 30). Two days after STZ injection (day +2), half of the mice (n = 10) received sustained-release insulin implants (LinBit[®]; insulin release rate: approximately 0.1 unit/day per pellet for over 30 days, 1 pellet per mouse) (LinShin Canada, Inc., Scarborough, Ontario, Canada)

(31, 32). Body weight and postprandial blood glucose (Glucometer, One Touch Ultra[®]; LifeScan Inc., Milpitas, CA, USA) were monitored 1 day prior to STZ injection (Before Treatment) and daily thereafter. Twenty-four-hour urine samples were collected on day -2 (Before Treatment), day +3, and day +6 using metabolic cages (Hatteras Instruments, Southbank Drive Cary, NC, USA). Water consumption was measured on day -2 (Before Treatment), day +3, and day +6. Seven days after STZ injection (day +7), all mice were sacrificed and kidney samples were harvested.

Enzyme-linked immunosorbent (ELISA) assays

Urinary neutrophil gelatinase-associated lipocalin (UNGAL), Alb, and AGT analyses were performed with ELISA kits according to the manufacturer's instructions. Using 24-h urine samples, the UNGAL concentration was examined by a mouse NGAL ELISA kit[®] (Cedarlane, Burlington, NC, USA) (33). UAlb excretion and UAGT excretion were measured using mouse albumin ELISA[®] (Immunology Consultants Laboratory, Inc., Newberg, OR, USA) (34) and the mouse total angiotensinogen assay kit[®] (Immuno-Biological Laboratories Co., Ltd., Takasaki) (21), respectively. The ratio of UAGT to UAlb (UAGT/UAlb) was calculated using each data set ($\text{ng} \cdot \mu\text{g}^{-1}$). Using urinary excretion levels of Alb, AGT, and UAGT/UAlb ratio, we calculated the %change in UAlb, %change in UAGT, and %change in UAGT/UAlb using the following formula:

$$[(\text{parameter on day +3 or on day +6} / \text{parameter of Before Treatment}) - 1] \times 100$$

Urinary protein (UPro) assay

UPro excretion over a 24-h period was evaluated using the Bradford protein assay (Bio-Rad, Hercules, CA, USA) (35). As a standard, rat serum protein (Chondrex, Redmond, WA, USA) was used.

Quantitative real-time RT-PCR

RNAs were extracted from the tissues using the RNeasy Mini kit[®] (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Expression of the AGT gene in the kidney was examined using the Brilliant II QRT-PCR Master Mix kit, 1-Step[®] (Stratagene, Santa Clara, CA, USA) following the RT-PCR conditions according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed as previously described (36, 37). Data from quantitative real-time RT-PCR were normalized to β -actin mRNA expression. Primer sequences were as follows: AGT, forward primer 5'-TAT CCA CTG ACC CAG TTC TT-3', reverse primer 5'-AAG TGA ACG TAG GTG TTG AAA-3', probe 5'-/6-FAM/CTG TGA CAG GGT GGA

AGA TGA ACT TGC CA/3BHQ-1/3'; β -actin, forward primer 5'-ATC ATG AAG TGT GAC GTT GA-3', reverse primer 5'-GAT CTT CAT GGT GCT AGG AGC-3', probe 5'-6-FAM/TCT ATG CCA ACA CAG TGC TGT CTG GT/3BHQ-2/3'.

Immunohistochemical (IHC) staining

The level of expression of AGT protein in the kidneys was examined by IHC as described previously (37, 38). Renal sections were fixed in 4% paraformaldehyde, embedded in paraffin, and deparaffinized in xylene. Then, 3- μ m-thick tissue sections were prepared by an out-sourcing company (Mass Histology Service, Worcester, MA, USA). IHC was performed with an automated system (Autostainer; Dako, Carpinteria, CA, USA) and slides were counterstained with hematoxylin. The primary antibody against rat polyclonal AGT was obtained from Immuno-Biological Laboratories Co., Ltd. Semi-quantitative analysis of the level of immunoreactivity was evaluated in a blind manner using a semi-automatic image analysis system that employed the Image-

Pro plus software (Media Cybernetics, Bethesda, MD, USA) as previously described (37–40).

Statistical analyses

All data are presented as the means \pm S.E.M. Data of the animal profiles, urine samples, and AGT expression were evaluated by the one-way repeated-measures ANOVA or one-way factorial ANOVA with Dunnett's test as a post-hoc test. $P < 0.05$ was considered to be statistically significant. All of the computations, including data management and statistical analyses, were performed with JMP software (SAS Institute, Cary, NC, USA).

Results

Animal profiles

The postprandial blood glucose levels and body weights of the mice are shown in Fig. 1. The postprandial blood glucose levels were elevated 1 day after STZ injection with respect to those in the control group. Two days

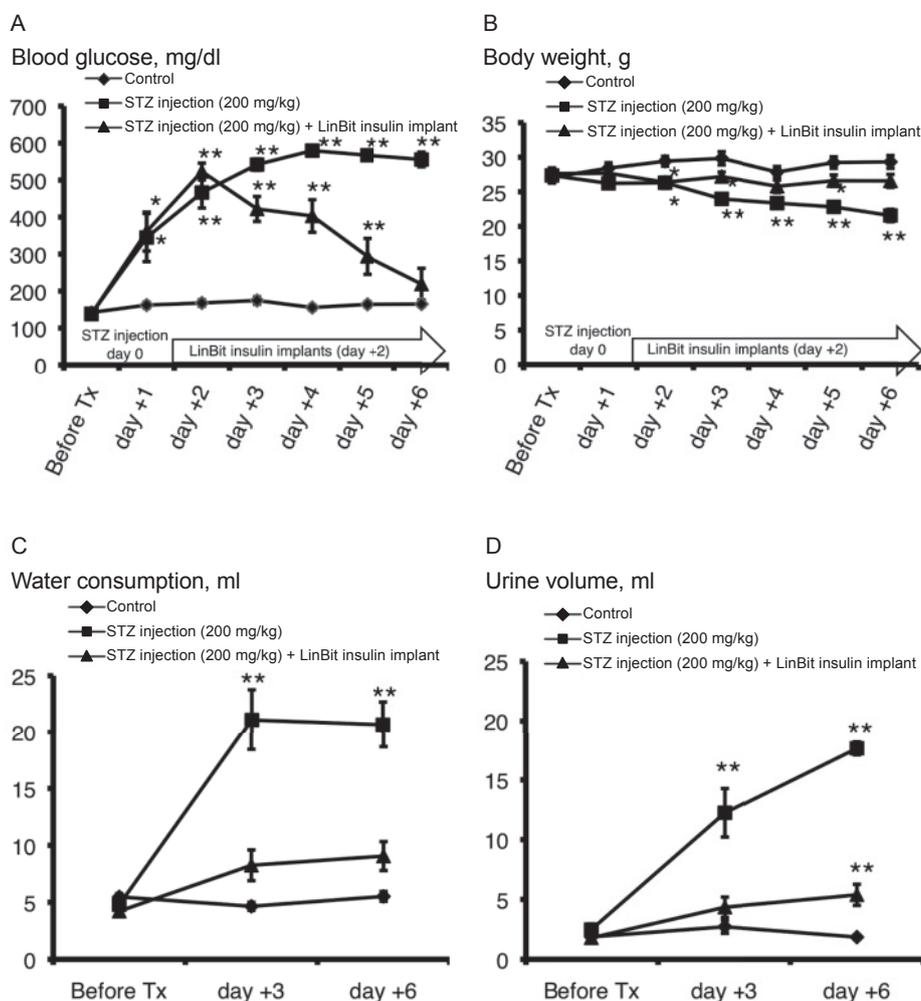


Fig. 1. Changes in postprandial blood glucose (A), body weight (B), water consumption (C), and urine volume (D) in control, STZ-induced type 1 diabetic, and LinBit insulin-treated diabetic mice before and after a single intraperitoneal injection of STZ. Data are expressed as the mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. control mice. STZ, streptozotocin.

after STZ injection, all mice were diabetic (> 400 mg/dl) (30). The change in postprandial blood glucose was relieved by insulin treatment during the experiment (Fig. 1A). Body weights of the diabetic mice were significantly lower than those of the control mice. Insulin treatment offset this change (Fig. 1B).

Water consumption and urine volume

Water consumption by mice of the STZ group was markedly greater than that of the control group 3 and 6 days after STZ injection ($P < 0.01$ vs. control). This effect was relieved by insulin treatment (Fig. 1C). The 24-h urine volume in mice of the STZ group was markedly greater than that of the control group 3 and 6 days after STZ injection ($P < 0.01$ vs. control). The change in urine volume was mitigated by insulin treatment (Fig. 1D).

Measurement of UNGAL concentration and temporal profiles of UAlb, UAGT, and UPro excretions

It is known that STZ occasionally induces acute kidney injury (AKI). Before the assays using urine samples, the

UNGAL concentration (ng/ml) was measured, as it may be used as an early biomarker of AKI (cut-off line: UNGAL concentration higher than 200 ng/ml) (41). As a result, all mice used in the assays were precluded of AKI (urine samples on day +6: control group, 35 ± 7 ng/ml; STZ injection group, 24 ± 4 ng/ml; STZ injection + LinBit insulin implant group, 46 ± 12 ng/ml).

Using urine samples from mice that were non-exhibitive of AKI, UAlb, UAGT, and UPro excretion were measured. Before STZ injection and on day +3 after STZ injection, UAlb excretion was not different among the 3 groups. However, UAlb excretion increased 6 days after STZ injection (130.0 ± 31.8 μ g/day) with respect to the controls (52.5 ± 8.4 μ g/day) ($P = 0.0159$). This change was completely negated by insulin treatment (40.8 ± 5.8 μ g/day) (Fig. 2A). UAGT excretion was higher in the STZ group (249.2 ± 63.5 μ g/day) than in the control group (19.7 ± 4.9 μ g/day) ($P = 0.0006$) 3 days after STZ injection, and it continued to increase in the STZ group (349.6 ± 89.1 μ g/day) while the control group (15.9 ± 2.2 μ g/day) stayed constant 6 days after STZ injection

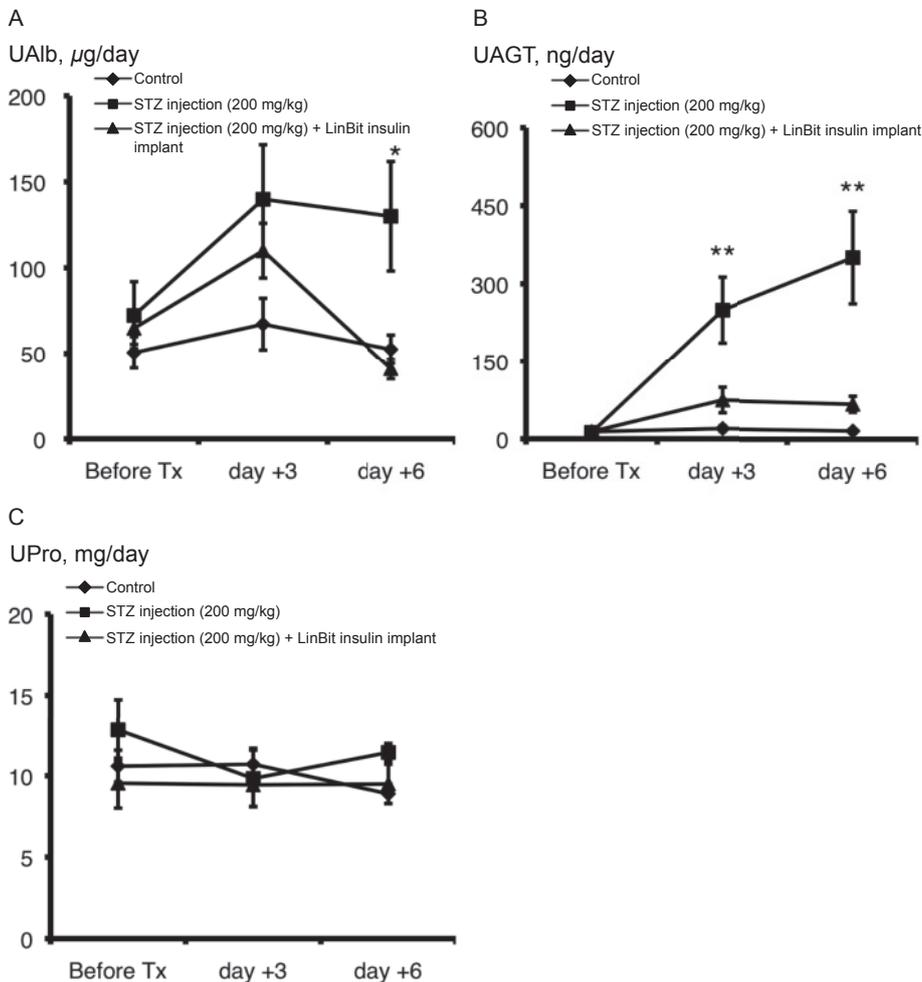


Fig. 2. Changes in UAlb (A), UAGT (B), and UPro (C) excretion in control, STZ-induced diabetic, and LinBit insulin-treated diabetic mice. Urine samples were collected before STZ injection and on days +3 and +6 after STZ injection. Data are expressed as the mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. control mice. UAlb, urinary albumin; UAGT, urinary angiotensinogen; UPro, urinary protein; STZ, streptozotocin.

($P = 0.0002$). This change was also completely negated by insulin treatment (on day +3: $74.5 \pm 24.6 \mu\text{g}/\text{day}$, on day +6: $67.3 \pm 15.5 \mu\text{g}/\text{day}$) (Fig. 2B). UPro excretion was not significant in all groups (Fig. 2C).

Ratio of UAGT/UAlb and the %change in UAlb, UAGT, and UAGT/UAlb

The UAGT/UAlb ratio ($\text{ng}\cdot\mu\text{g}^{-1}$) was statistically higher in the STZ group than in the control group 3 and 6 days after STZ injections ($P < 0.01$) (Fig. 3A). This effect was completely mitigated by insulin treatment. Although the %change in UAlb did not differ significantly between the control and STZ groups 3 days after STZ injection, the %change in UAlb increased 6 days after STZ injection ($131.2\% \pm 46.3\%$) with respect to the controls ($16.9\% \pm 13.0\%$) ($P = 0.0105$). This change was completely abolished by insulin treatment ($-31.9\% \pm 8.6\%$) (Fig. 3B). The %change in UAGT was higher in the STZ group ($1875.7\% \pm 657.7\%$) than in the control group ($91.2\% \pm 53.9\%$) ($P = 0.0044$) 3 days after STZ injection and continued to increase in the STZ group

($3985.9\% \pm 1362.4\%$) with respect to the control group ($76.5\% \pm 41.6\%$) ($P < 0.00001$) 6 days after STZ injection. This change was also completely abolished by insulin treatment (on day +3: $476.5\% \pm 179.0\%$, on day +6: $385.1\% \pm 112.9\%$) (Fig. 3C). The %change in UAGT/UAlb ratio was higher in the STZ group ($892.2\% \pm 297.9\%$) than in the control group ($64.1\% \pm 52.9\%$) ($P = 0.0046$) 3 days after STZ injection and continued to increase in the STZ group ($2103.0\% \pm 555.0\%$) with respect to the control group ($60.7\% \pm 37.8\%$) 6 days after STZ injection ($P = 0.0007$). This change was also completely abolished by insulin treatment (on day +3: $242.8\% \pm 95.7\%$, on day +6: $742.7\% \pm 213.9\%$) (Fig. 3D).

AGT mRNA expression in the kidneys of STZ-induced diabetes with or without insulin treatment

Kidney samples harvested 7 days after STZ injection were used to detect AGT mRNA levels. Increased AGT mRNA expression in the kidneys was observed in diabetic mice ($P < 0.01$), and this increase was negated with insulin treatment (Fig. 4A).

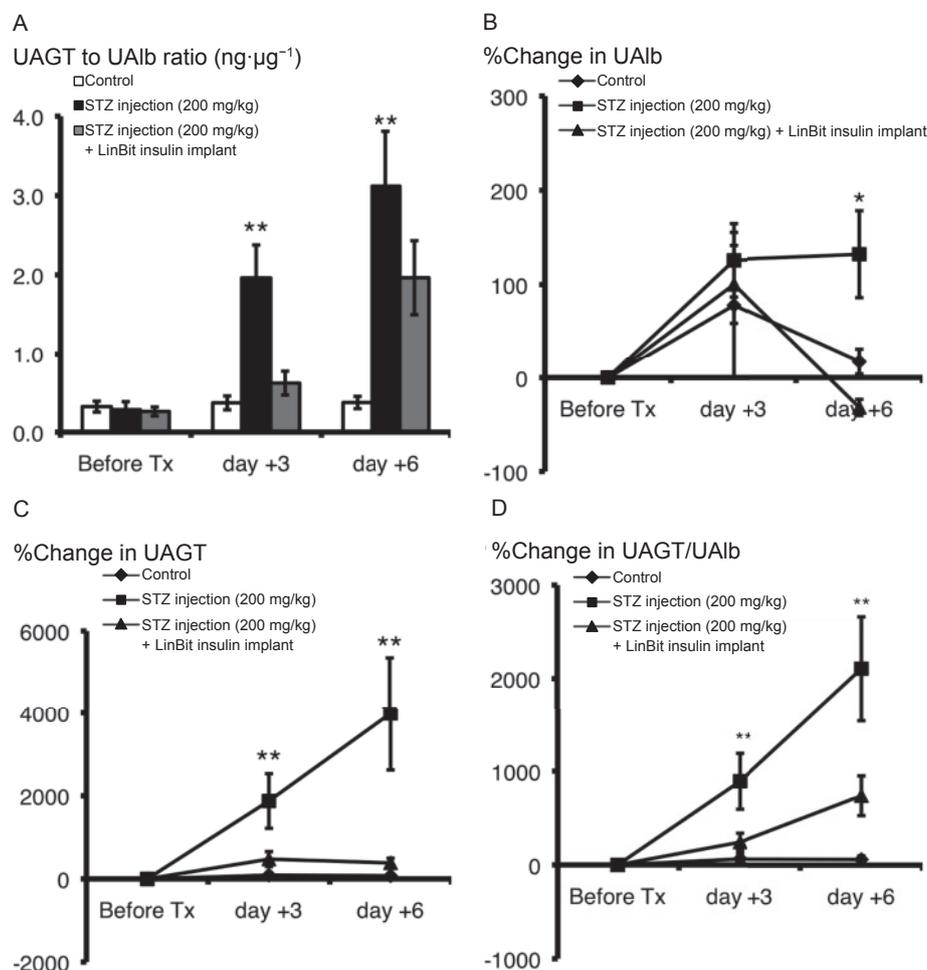
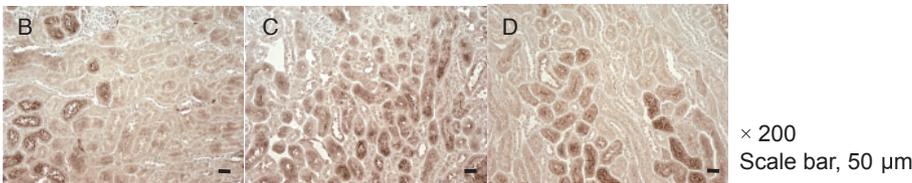
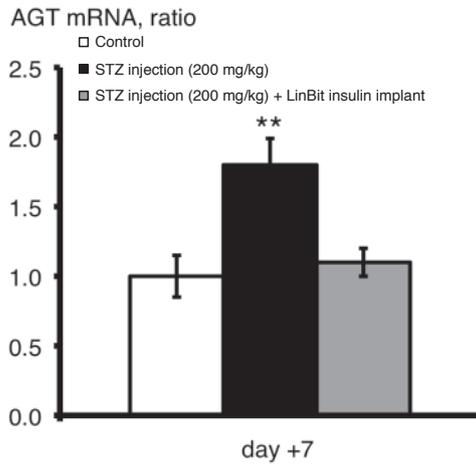


Fig. 3. Ratio of UAGT/UAlb ($\text{ng}\cdot\mu\text{g}^{-1}$) (A) and %change in UAlb (B), UAGT (C), and UAGT/UAlb (D) in control, STZ-induced diabetic, and LinBit insulin-treated diabetic mice were calculated using urinary excretion data. Data are expressed as the mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. control mice. UAGT, urinary angiotensinogen; UAlb, urinary albumin; STZ, streptozotocin.

A



E

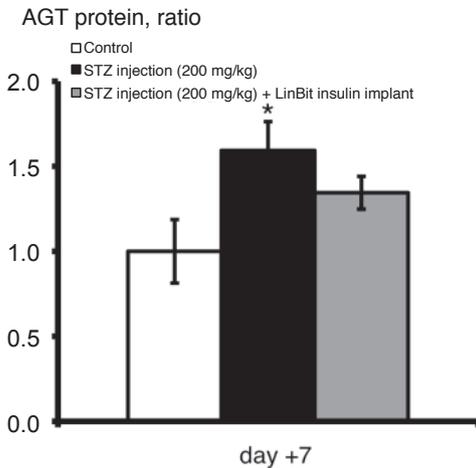


Fig. 4. AGT mRNA expression (A) and immunohistochemical localization of AGT protein (B, C, D, and E) in the kidneys of control, STZ-induced diabetic, and LinBit insulin-treated diabetic mice. We used kidneys on day +7 after STZ injection. Immunohistochemical staining (magnification, $\times 200$; scale bar, $50 \mu\text{m}$) shows increased AGT in the kidneys of diabetic mice (C) compared with control (B) or LinBit insulin-treated diabetic mice (D). AGT protein expression levels are indicated in panel E. Data are expressed as the mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. control mice. AGT, angiotensinogen; STZ, streptozotocin.

AGT protein expression in the kidneys of STZ-induced diabetes with or without normalization with insulin treatment

Kidney samples harvested 7 days after STZ injection were used to detect AGT protein levels. IHC staining for AGT protein (Fig. 4: B, C, and D) was clearly of greater intensity in the kidneys (especially in the proximal tubules) of diabetic mice than in those of control mice ($P < 0.05$), and this effect was mitigated by insulin treatment (Fig. 4E).

Discussion

In this study we demonstrate that UAGT and UAlb excretion is elevated in STZ-treated T1DM mice. The following novel findings emerged: UAGT excretion accompanied by the intrarenal AGT expression increase earlier than UAlb after STZ injection compared to controls. Moreover, the UAGT to UAlb ratio is increased in diabetic mice compared to control mice. Our data revealed conclusive evidence that UAGT is an early biomarker of increased RAS in T1DM.

In our study we have used STZ to induce T1DM. STZ is widely used to induce type 1 diabetes in rodent models

(30, 42, 43). However, it is known that in some cases STZ can induce AKI. STZ is an unusual aminoglycoside containing a nitrosoamino group, which can cause acute toxic injury to the kidney (44). Therefore, before the assays using urine samples, the UNGAL concentration was measured to detect AKI. NGAL concentration is usually used as an early biomarker of AKI (41, 45). The mice used in this study did not exhibit AKI, only exhibiting the expected signs of STZ-induced type 1 diabetes.

UAGT excretion is higher in patients with type 1 diabetes compared to control subjects (28). Despite the importance of the RAS in the development of DN, the significance of intrarenal RAS, especially the role of AGT, in the early stage of T1DM is not yet fully elucidated. Here, we investigated UAGT level as a candidate marker of activation of the RAS in type 1 diabetes. STZ-induced type 1 diabetic mice showed typical symptoms of diabetes mellitus and insulin treatment ameliorated these changes in agreement with short-term investigation studies (46, 47). After development of type 1 diabetes, we observed increases in excretion levels of UAlb and UAGT in diabetic mice compared to control and insulin-treated groups. These observations indicate that urinary excretions of AGT and Alb were increased in the STZ-treated mice compared to the control animals. An important observation is that the augmented excretion level of AGT in the urine was apparent before the development of increased Alb levels. This could be because the UAGT level is highly sensitive to the onset of nephropathy. Furthermore, our data showed that the ratio of UAGT to UAlb was significantly higher in the STZ group than in the control group. Because insulin treatment mitigated the increased excretion of UAlb and UAGT, it is thought that increased glucose level induced elevated UAlb and UAGT excretions. Although we have measured a late-appearing marker of kidney injury, UPro excretion, we did not find any significant difference between groups. Our results indicate that augmented UAGT level might potentially serve as a novel, early biomarker of intrarenal RAS activation in experimental type 1 diabetes. These findings could lead to earlier diagnosis of diabetic complications and start of medical treatment. They also suggest that we should re-assess the clinical significance of RAS blockade.

In the investigation of biomarker using urinary samples, the suspicion that enhanced UAGT excretion in a diabetic model is just a non-specific consequence of damaged glomerular filtration barrier remains. To address this issue, we monitored AGT expression in the kidneys. AGT mRNA and protein expression in the kidneys, especially in the proximal tubules, were increased in the STZ group compared to the control group. This finding is congruent with previous reports demonstrating that intrarenal AGT

is formed primarily in the proximal tubule cells and is enhanced in diabetic conditions (17, 18, 21, 48, 49). Although the detailed localization of AGT mRNA and protein in the convoluted and straight proximal tubules has not been yet clarified (50 – 54), our recent data suggest that AGT mRNA in the straight tubules and AGT protein in the convoluted and straight proximal tubules are important and need to be investigated in future studies (55). The data in the kidneys may rule out the possibility of a damaged glomerular filtration barrier. Because these increased mRNA and protein levels were ameliorated in the insulin-treated group, we suggest that increased blood glucose induces intrarenal AGT mRNA and protein expression and a subsequent increase in UAGT excretion.

The pathogenesis of DN is clearly multifactorial (5, 6, 56, 57). The activation of the intrarenal RAS has been shown to have a potential role in the mechanism of DN (6, 19, 29, 58 – 60), as shown in large-scale clinical trials (8 – 13). Recently, the Randomized Olmesartan and Diabetes Microalbuminuria Prevention (ROADMAP) study (13) provided novel insights that olmesartan, an ARB, was associated with delayed onset of microalbuminuria, even though blood pressure control in the patients with type 2 diabetes was excellent. This result suggests the possibility of preemptive treatment of ARB for the delay or prevention of microalbuminuria in diabetes (14). Based on the outcome of this large-scale clinical trial, we expect the intrarenal AGT to have an important role in the development of DN because we already reported that ARB treatment suppressed intrarenal and urinary AGT in animal studies (16, 61) as well as in clinical studies (27, 62).

Various proteins, such as urinary type IV collagen, adiponectin, and advanced glycation end products, have been implicated as potential markers of DN (63 – 65). However, such divergent findings have complicated the search for a reliable biomarker. AGT is the only known substrate for renin, which is the rate-limiting enzyme of the RAS (23, 24). Upregulation of AGT levels may lead to elevation of the angiotensin peptide levels (25, 26). Our recent studies in diabetic models have documented the involvement of AGT in activation of the RAS (16, 27, 28). While intrarenal renin expression is also increased in diabetic animals (16), some *in vitro* reports (66 – 72) supported that high glucose augmented AGT gene expression using a cell line of rat immortalized renal proximal tubular cells. Using the same cell line, it was also reported that the insulin treatment inhibited the stimulatory effect of high glucose on the expression of AGT. Thus, AGT plays an important role in the mechanism of high glucose-induced AGT expression and the development of diabetic complications including

nephropathy.

A shortcoming of this study is the exclusion of a long-term study for the role of AGT in the development and progression of DN. However, it was repeatedly shown that STZ-induced T1DM leads to DN in long-term studies (29 – 32). Here, we focused on the initial increase of UAGT in a short-term study and showed that increased UAGT may signal the development of RAS. Moreover, we have reported on the augmented AGT expression and RAS activation in the progression of DN (16 – 18). Based on our previous data and the results of this study, we are projecting future clinical studies to focus on the role and mechanisms of AGT in the onset of nephropathy and the progression of kidney dysfunction, as well as their relationships.

Acknowledgments

This study was supported by grants from National Institute of Diabetes and Digestive and Kidney Diseases (R01DK072408 to HK), National Center for Research Resources (P20RR017659 to HK), American Heart Association (10GRNT3020018 to HK and 10GRNT 4540000 to AZ), and National Institute of Child Health and Human Development [Tulane Building Interdisciplinary Research in Women's Health (BIRCWH), K12HD043451 to AZ]. The authors acknowledge scientific discussion and/or excellent technical assistances from L. Gabriel Navar, Ph.D.; Maki Urushihara, M.D., Ph.D.; Andrei V. Derbenev, Ph.D.; Hong Gao, Ph.D.; Salem I. Elkhayat, M.S.; and Ayumi Kitano, M.S. (Tulane University).

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