Regulation of urinary ACE2 in diabetic mice

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Recent work within the renin-angiotensin system has focused on angiotensin-converting enzyme-2 (ACE2) (1, 3, 7, 8, 18, 19, 21, 25, 27, 35, 36, 38, 40, 43, 46–48). This enzyme, which is highly expressed in the kidney, efficiently metabolizes ANG II, an action that seems critically important in preventing the accumulation of ANG II, and promotes the formation of ANG-(1–7) (6, 28, 30, 37, 42). Overactivity of the renin-angiotensin system (RAS) is felt to play an important role in the development of diabetic nephropathy. A line of evidence in support of the view that the RAS is overactive in diabetes comes from the effectiveness of ACE inhibitors and ANG II blockers in decreasing proteinuria and delaying the progression of kidney disease. The therapeutic response, however, is not homogenous with responders and nonresponders, consistent with the notion of different levels of kidney RAS activity interindividually (5).

The ultimate fate of whether there is ANG II overactivity in diabetic kidney disease and other cardiovascular pathologies may depend on genetic and acquired factors (23). For instance, changes in ACE activity which may be, in part, genetically determined may favor or attenuate ANG II formation and diabetic kidney disease in mice models (9). The degradation of ANG II is likewise critically important for the steady-state levels of this peptide. Measuring ANG II, however, is not the answer because the levels of ANG II in plasma are usually not elevated, and ANG II levels in the urine are highly variable and not very useful because this peptide is degraded rapidly intrarenally and later on in the urine which contains many peptidases (1).

We reasoned that urinary ACE2 could be a marker of the development of diabetic nephropathy and, moreover, could reflect the activity of RAS within the kidney. Recently urinary ACE2 has been found to be increased in urine of diabetic animals (2, 41) and diabetic patients (17, 44) but the mechanism of this increase is largely unknown. Moreover, very little is known regarding the physiological regulation of kidney ACE2 activity. Here we show that urinary ACE2 activity in db/db and streptozotocin (STZ)-induced model of diabetes is markedly increased and report the effect of various maneuvers on the activity and protein expression of this enzyme in the urine of control and diabetic mice.

METHODS

Mouse animal models. All studies were conducted with the review and approval of the Institutional Animal Care and Use Committee. As a model of type 2 diabetes female db/db mice (C57BLKS/JLepr) were used. Their lean age-matched littermates (db/m) served as nondiabetic controls.

As db/db mice are resistant to insulin (12, 31), streptozotocin (STZ)-treated mice were used to study the effect of glycemic control by insulin on urinary ACE2. Diabetes was induced in male C57BL/6J mice by intraperitoneal injections of STZ (Sigma-Aldrich, St. Louis, MO), at a dose of 150 mg/kg body wt in sterile 0.05 M sodium citrate (pH 4.5) (32). STZ-treated mice were randomly divided into two groups: one group received insulin pellets for 12 wk (Linbit, LinShin, MO), at a dose of 0.1–0.2 IU/mouse per day) and the other group of STZ mice did not receive insulin.

Blood was obtained from the tail vein and glycemia was assessed using One Touch Ultra Glucometer (LifeScan, Mountain View, CA). Urinary albumin and creatinine were measured using commercially available kits (Exocell, Philadelphia, PA).

Male wild-type (WT) and ACE2 knockout (ACE2KO) mice on C57BL/6J background (6) (breeding pairs donated by Drs. S. Gurley and T. Coffman, Duke University, Durham, NC) were used to obtain urine for validation of urinary ACE2 activity, to examine specificity of

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ACE2 immunoreactive protein bands in Western blot, and to perform ANG II-degradation studies.

Pharmacological agents and diets. Starting at 7 wk of age, db/m (n = 10) and db/db mice (n = 10) were followed for several months to examine the effect of age on parameters such as urinary ACE2 and albumin/creatinine ratio. Another group of db/m and db/db mice were followed for 4 wk the same way, and then were assigned to drink tap water with an ACE inhibitor, captopril (n = 7–10 in each group), at a dose of 60 mg·kg⁻¹·day⁻¹ for several weeks. After 1 wk of washout, mice received tap water with an ANG II receptor antagonist, telmisartan (Boehringer Ingelheim), at a dose of 2 mg·kg⁻¹·day⁻¹ (n = 7) for several weeks. During the entire period, spot urine was collected repeatedly every 1–4 wk, so that under control conditions and at each treatment period at least four spot urine collections were obtained.

The effect of salt intake on urinary parameters was examined by providing high-salt (8% NaCl) and low-salt (0.1% NaCl) diets (Harland Teklad) for several weeks to female db/m and db/db mice.

Separation of the glomeruli from renal tubules using magnetic beads. Mice were anesthetized by intraperitoneal injection of pentobarbital and perfused with 8 × 10⁷ particles of Dynabeads M-450 (Invitrogen) diluted in 40 ml of PBS. Mouse kidneys were then removed and processed as described previously (34). Briefly, kidneys were minced into small pieces and digested in collagenase A (Roche) and deoxyribonuclease I (Sigma) solution at 37°C for 30 min with gentle agitation. The digested tissue was pressed through a 100-m and deoxyribonuclease I (Sigma) solution at 37°C for 30 min with

Urine obtained. Mice were monitored for several weeks. After 1 wk of washout, mice received tap water with an ANG II receptor antagonist, telmisartan (Boehringer Ingelheim), at a dose of 2 mg·kg⁻¹·day⁻¹ (n = 7) for several weeks. During the entire period, spot urine was collected repeatedly every 1–4 wk, so that under control conditions and at each treatment period at least four spot urine collections were obtained.

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Glomeruli were homogenized in a buffer [(in mmol/l) 50 HEPES, pH 7.4, 150 NaCl, 0.5% Triton X-100, 0.025 ZnCl₂, and 1.0 PMSF] for overnight at 4°C. The glomeruli containing Dynabeads were collected by a magnetic particle concentrator (Dynal AS, Norway). The portion of digested kidney tissue deprived of glomeruli was considered to represent tubular fraction.

Isolated glomeruli in PBS suspension were viewed under the microscope and the purity of the glomeruli was established to be ≥95%. Glomeruli were homogenized in a buffer ([in mmol/l] 50 HEPES, pH 7.4, 150 NaCl, 0.5% Triton X-100, 0.025 ZnCl₂, and 1.0% PMSF) for overnight at 4°C. The precipitate was removed by centrifugation at 5 ml of HBSS. The resulting cell suspension was then centrifuged at 200 xg for 5 min at 4°C. The glomeruli containing Dynabeads were collected by a magnetic particle concentrator (Dynal AS, Norway). The portion of digested kidney tissue deprived of glomeruli was considered to represent tubular fraction.

ACE2 activity. Urine, serum, and kidney ACE2 activity were determined following incubation with the intramolecularly quenched synthetic ACE2-specific substrate Mca-APK-Dnp (Anaspec). The measurements were performed in black microtiter plates with a 100 µl total volume. Briefly, 2 µl urine, 2 µl serum or 1 µg total protein from tissue homogenate (renal cortex, isolated glomeruli or tubular fraction) was added to wells containing a buffer (50 mmol/l 4-morpholineethanesulfonic acid, 300 mmol/l NaCl, 100 µmol/l ZnCl₂, and 0.01% Triton-X-100, pH 6.5), containing EDTA-free tablets (Roche) and 10 µmol/l substrate. Reactions were in duplicates (one of two wells constituted a blank). Blank wells contained the same components, but 10 µmol/l of a specific ACE2 inhibitor, MLN-4760 (gift from Millenium Pharmaceuticals, Cambridge, MA), was also added.

After incubation at ambient temperature for 1 h (kidney cortex) or 16–24 h (serum and urine), fluorescence was measured using an FLX800 microplate fluorescence reader (BIOTEK Instruments, Winooski, VT) at 320 nm excitation and 420 nm emission wavelength. Total fluorescence was corrected for volume (in serum samples) or protein content (in tissue homogenates) after subtracting blank values.

At 420 nm, mouse urine by itself generates a detectable signal which may obscure fluorescence stemming from ACE2-specific substrate (Mca-APK-Dnp) hydrolysis. To account for this interference we used specific environment of the urine to examine detectability levels of mouse recombinant ACE2 protein in the ACE2 activity assay. Urine obtained from ACE2 knockout mice was spiked with increasing amounts of purified soluble mouse rACE2 which resulted in an increase in fluorescence signal in a dose-dependent manner with a linear relationship ranging from 15.6 to 1000 pg/ml. The range of ACE2 activity in urine from wild-type mice corresponded to concentrations of 454–1487 pg/ml urine by the comparison with purified recombinant enzyme under identical conditions. As little as 15.6 pg/ml of rACE2 was detectable in spiked urines from ACE2 knockout mice, suggesting that ACE2 protein levels in normal urines are far-off the detection limit of the ACE2 activity assay, which warrants its use for quantitative purposes.

Western blot. Kidney cortex total and membrane protein fractions were extracted as previously described (43, 47).

For Western blot of urines, urine samples were concentrated on centrifugal concentrators of 5-kDa cut off. Proteins were separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked in nonfat dry milk solubilized in Tris-buffered saline solution containing 0.1% Tween 20 (7% wt/vol). The nitrocellulose membranes were incubated with primary anti-ACE2 antibody (R&D Systems or Abcam) and horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Bands were visualized using chemiluminescence system (Super Signal Pico, Pierce). For comparison of urinary ACE2 bands between control and diabetic mice, several gels were used in which a similar number of samples from each group was included. The average value of integrated density measured in db/m mice was set as 100% for each gel and the results for db/db mice were expressed as percentage of that measured in db/m controls. Variation from the mean was presented to reveal the level of variability in each group.

Exogenous ANG II degradation by the urine. Urine from male ACE2KO and WT mice matched for creatinine concentrations and equal amounts of total protein (1 mg) were individually incubated with 10⁻¹⁰ M ANG II (Sigma) by constant shaking for up to 8 h. In similar experiments, urines collected from 16-wk-old female db/m and db/db mice representing equal creatinine concentration (10 µg/ml) were incubated with ANG II (10⁻⁸ M) for up to 4 h at 37°C in PBS pH 7.4 containing 5 µM ZnCl₂. The incubation was performed with or without addition of the specific ACE2 inhibitor, MLN-4760 (10⁻⁶ M). Following the incubation, samples were diluted 1/5 in EDTA-containing buffer (0.1 M phosphate buffer, pH 7.4 containing 0.15 M NaCl, 1 mM EDTA, 0.1% BSA) and immediately frozen. The quantity of ANG II in the diluted samples was measured using a solid-phase immobilized epitope immunoassay kit (SPIObio, Cayman Chemical, Ann Arbor, MI). ANG II quantity in each sample was expressed as a percentage of the initial ANG II load (100%). This EIA method shows less than 0.1% cross-reactivity with ANG-1 (7). However, ANG III, ANG IV, and ANG-(4–8) exhibit 36%, 33% and 41% cross-reactivity, respectively, expressed in terms of reactivity percent toward ANG II (39).

Administration of soluble recombinant ACE2. Female db/m (n = 5) and db/db (n = 5) at 10 wk of age were used to study the effect of rACE2 administration on serum and urinary ACE2 activity. Before administration of rACE2, blood was collected from tail vein (around 15 µl). Immediately after voiding urine, mice were administered an intraperitoneal bolus of recombinant ACE2 (1 mg/kg). Urine was then collected in metabolic cages over 3 h after rACE2 intraperitoneal bolus and blood was taken from tail vein at 3 h after rACE2 administration.

Statistical analysis. Changes over the course of the experiments between the groups were analyzed using general linear model (GLM) on the SPSS Statistics 20 software (IBM). Differences between two groups were analyzed using a two-tailed Student’s t-test, and for nonnormally distributed data, a nonparametric Mann-Whitney test was used. For multiple comparisons ANOVA was used followed by LSD post hoc test. A Pearson’s test was used for correlations between two variables. For data with normal distribution, values were expressed as means ± standard error of the mean (SE). For nonnormally distributed data, the median and interquartile range were used. For
calculation of half-lives of ANG II one phase decay analysis was used (GraphPad Prism).

RESULTS

ACE2 activity in urine from diabetic (db/db mice) and controls (db/m mice). At 7 wk of age, db/db mice have been diabetic for 2 wk (31). Urinary ACE2 activity at this early age was already twofold higher compared with db/m (n = 10) [23 ± 4 vs. 11 ± 0.5 relative fluorescence units (RFU) μg creatinine⁻¹·h⁻¹, P < 0.01, respectively]. At this early age, the albumin/creatinine ratio, by contrast, was not significantly different between db/m (n = 10) and db/m (n = 10) (58 ± 12 vs. 45 ± 7 μg/mg, P = NS, respectively).

During several consecutive spot urine measurements from 7 to 25 wk of age, urinary ACE2 activity/creatinine ratio in db/db mice increased over time and was consistently and significantly higher than in db/m mice of the same age (Fig. 1). Albumin/creatinine ratio also increased with age in db/db but not in db/m mice (Fig. 1B). The increase in ACE2/creatinine ratio ante-dated the increase in albumin/creatinine ratio (compare Fig. 1, A and B). When two to three adjacent measurements were averaged at each time interval, the differences in urinary ACE2 activity/creatinine ratio (Fig. 1C) and albumin/creatinine ratio (Fig. 1D) became even more apparent.

When measured as excretion rate (12 h urine collection in mice housed in metabolic cages), urinary ACE2 activity was also increased in urines from db/db mice of about 18 wk of age compared with age-matched db/m mice (Fig. 2A). ACE2 activity/creatinine ratio was also increased to about the same extent (Fig. 2B). ACE2 activity/creatinine ratio correlated well with ACE2 activity expressed per 12-h time unit when data from db/m and db/db mice were pooled for regression analysis (R = 0.818, P < 0.01, n = 10) (Fig. 2C). Urinary ACE2 activity, either expressed as 12-h excretion rate or as urine ACE2/creatinine ratio, correlated positively with 12-h urine volume (R = 0.912, P < 0.01, and R = 0.858, P < 0.01, respectively) (Fig. 2, D and E). A significant positive correlation (R = 0.679, P < 0.01) was also observed between albumin/creatinine ratio and ACE2 activity measured from spot urines collected from db/m (n = 20) and db/db mice (n = 20) over a course of 20 wk (18–38 wk of age) (Fig. 2F).
Urinary ACE2 activity during changes in dietary salt intake.

Urinary ACE2 activity was measured at weekly intervals four consecutive times in mice on normal salt (0.49% NaCl), high salt (8% NaCl) and low salt (0.1% NaCl) salt in their diets (Fig. 3A). The values from four consecutive ACE2 activity measurements were also averaged for each experimental group (Fig. 3B). During high-salt diet urinary ACE2 activity increased in both db/m and db/db groups compared with respective groups receiving normal-salt diet (Fig. 3, A and B). Under all these dietary conditions, db/db had higher levels of urinary ACE2 activity than their age-matched db/m controls. The low-salt diet ingestion in db/db mice caused the urine ACE2 activity to fall below the level of the ACE2 activity measured at the time of normal salt intake (P < 0.05). In db/m mice, low salt also reduced urinary ACE2 activity compared with a high-salt diet (P < 0.05). The differences in ACE2 activity between control and diabetic mice persisted during changes in dietary salt intake (Fig. 3).

In a separate set of db/db mice that received high or low salt concurrently over several weeks, there was a significantly higher ACE2 activity in urines from mice kept on high-salt than those on low-salt diet (112 ± 30 vs. 23 ± 6 RFU·µg creatinine⁻¹·h⁻¹, respectively, P < 0.05). Kidney ACE2 activity, however, was not significantly different between high- and low-salt diets (126 ± 9 vs. 110 ± 15 RFU·µg creatinine⁻¹·h⁻¹, respectively, P = NS). Urinary ACE2 activity during captopril and telmisartan administration. Whether RAS inhibition affects urinary ACE2 activity was examined in db/m and db/db mice during the continuous administration of an ACE inhibitor, captopril, and a specific AT1 receptor blocker, telmisartan. In both db/m and db/db mice urinary ACE2 activity/creatinine ratio did not differ from ACE2 activity/creatinine ratio measured longitudinally in db/m and db/db mice before and during captopril administration daily (Fig. 4A). The AT1 receptor blocker, telmisartan, also did not alter significantly urinary ACE2 activity/creatinine ratio either in the db/m or the db/db mice (Fig. 4A). Accordingly, the difference between db/m and db/db persisted over the time course of the administration of captopril and telmisartan (Fig. 4A). The lack of a significant effect of these two RAS blocking agents on urinary ACE2 activity/creatinine ratio was
A significant increase in urinary ACE2 activity during high-salt diet intake in normal salt (control) was no significant difference between low- and normal-salt diet. *High salt vs. control* (Fig. 3A). When the values from 3–4 consecutive measurements were averaged for each experimental condition, however, in *db/db* mice the administration of both RAS blocking agents was associated with a significant decrease in albumin/creatinine ratio (*P < 0.05*) (Fig. 4D).

**Kidney as a source of elevated urinary ACE2 in *db/db* mice.** It has been previously shown that kidney cortex ACE2 protein and ACE2 activity are increased in *db/db* mice of various age groups compared with *db/m* (22, 43, 47). Serum ACE2 activity in *db/db* mice was significantly increased compared with *db/m* in young 8-wk-old mice (3.7 ± 0.2 vs. 2.6 ± 0.1 RFU·µl⁻¹·h⁻¹, *P < 0.01*, respectively, *n = 9* in each group) and in mice at 38 wk of age (1.2 ± 0.2 vs. 0.7 ± 0.1 RFU·µl⁻¹·h⁻¹, *P < 0.05*, *n = 9* and *n = 10*, respectively).

We wanted to ascertain whether the increase in urinary ACE2 in *db/db* mice originates from an increase in renal or from an increase in circulatory ACE2. To examine this issue soluble rACE2 was administered to *db/m* mice at a dose of 1 mg/kg. Administration of rACE2 (1 mg·kg⁻¹·day⁻¹) has been previously shown to produce a marked and reproducible increase in serum but not kidney ACE2 activity in C57BL6 mice (42). In *db/m* mice (*n = 5*) that received soluble recombinant (r)ACE2 acutely (1 mg/kg ip), serum ACE2 activity was markedly increased compared with the ACE2 activity in serum collected before rACE2 administration (181 ± 12 vs. 1.9 ± 0.6 RFU·µl⁻¹·h⁻¹, *P < 0.001*) (Fig. 5). ACE2 activity in urine, however, did not increase significantly after rACE2 infusion to *db/m* mice (9.5 ± 2.9 vs. 5.7 ± 0.3, *P = NS*, respectively) (Fig. 5).

As in *db/m* mice, rACE2 (1 mg/kg) administration to *db/db* mice resulted in a marked increase in serum ACE2 activity compared with the ACE2 activity in serum collected before rACE2 administration (222 ± 11 vs. 2.7 ± 0.7 RFU·µl⁻¹·h⁻¹, *P < 0.001*), but no significant increase in urinary ACE2 activity was observed (44 ± 11 vs. 75 ± 23 RFU·µg creatinine⁻¹·h⁻¹, *P = NS*, respectively) (Fig. 5).

Since a profound increase in serum ACE2 activity after rACE2 infusion does not translate into an increase in urinary ACE2 activity in control or diabetic mice with albuminuria, these results show that the primary source of ACE2 found in mouse urine is the kidney and not serum. This finding, moreover, suggests that the source of increased ACE2 in the urine of diabetic mice is of renal tubular origin rather than a consequence of altered glomerular permeability in diabetic mice.

**ACE2 enzymatic activity in *db/m* and *db/db* mice in isolated kidney tubules and glomeruli.** In kidney cortex, at 32 wk of age, ACE2 activity was significantly higher than in the *db/m* (76 ± 8 vs. 53 ± 6 RFU·µg protein⁻¹·h⁻¹, respectively, *P < 0.05*). To study whether the augmented urinary ACE2 activity in *db/db* mice is of glomerular or tubular origin, we assessed ACE2 enzymatic activity in isolated glomeruli and kidney tubules from 32-wk-old *db/db* mice. ACE2 activity measured in isolated renal tubular fractions from *db/db* mice was significantly higher than in *db/m* mice (99 ± 8 vs. 58 ± 3 RFU·µg

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**Fig. 3.** A: urinary ACE2 activity measured at 1- to 3-wk intervals several consecutive times in mice on a normal salt intake, on high-salt (8% NaCl), and on low-salt (0.1% NaCl) diet. The differences between *db/db* and *db/m* mice were significant by repeated measures using GLM analysis throughout the three experimental periods (*P < 0.001*). The four consecutive ACE2 activity measurements for each experimental period in A were averaged as shown in B. A significant increase in urinary ACE2 activity during high-salt diet intake in both *db/m* and *db/db* mice was observed compared with respective groups on a normal-salt diet. Compared with normal-salt diet, low-salt diet was associated with significantly lower ACE2 activity in *db/db* mice. In *db/m* mice there was no significant difference between low- and normal-salt diet. *High salt vs. normal salt (control) db/m (P < 0.05), #High salt db/db vs. normal salt (control) db/db and low salt db/db (P < 0.001). *Low salt db/db vs. normal salt (control) db/db (P < 0.05).*
protein $^{-1} \text{h}^{-1}$, respectively, $P < 0.001$) (Fig. 6A). ACE2 activity in isolated glomeruli was not significantly different between the control and diabetic mice ($9.0 \pm 4.6$ vs. $4.8 \pm 1.6$ RFU-$\mu$g protein $^{-1} \text{h}^{-1}$, respectively, $P = \text{NS}$) (Fig. 6B). In tubular fractions, levels of ACE2 activity per microgram total protein were on average 10 times higher than in isolated glomeruli (compare Fig. 6, A and B).

Kidney tubular ACE2 activity plotted against ACE2 activity measured in urines from the same mice obtained at the date animal was euthanized showed a significant positive correlation ($R = 0.639$, $P < 0.05$, $n = 13$). In contrast, no correlation was found between glomerular and urinary ACE2 activity in 32-wk-old $db/m$ and $db/db$ mice ($R = 0.079$, $P = \text{NS}$, $n = 14$). Altogether these patterns of glomerular and tubular ACE2 expression in $db/db$ mice suggest that the ACE2 activity increase seen in urines from diabetic mice is due to an increase in tubular rather than glomerular ACE2 because in $db/db$ mice ACE2 in the kidney is increased in tubules but not in glomeruli. In fact, in glomeruli ACE2 has been shown to be down-regulated by immunostaining in $db/db$ mice (48) and patients with type 2 diabetes (18).

**Urinary ACE2 activity in STZ-mice and effect of insulin.** To examine if the striking differences in urinary ACE2 in the $db/db$ mice were found in other model of diabetes, mice with STZ-induced diabetes were studied as well. Moreover, because $db/db$ mice are known to be insulin-resistant (12, 31), we used STZ-treated mice as a model to study whether insulin in itself, or by normalizing hyperglycemia or by improving kidney disease in diabetic mice, influences levels of urinary ACE2 activity.

Similar to $db/db$ model, in the STZ-treated mice urinary ACE2 activity was significantly increased compared with vehicle-treated controls throughout the follow-up (Fig. 7). Glycemia was reasonably well controlled in a group of STZ-mice that received insulin compared with vehicle controls (Fig. 8A). Urinary ACE2 activity/creatinine ratio in STZ mice receiving insulin was not significantly different from STZ-treated mice without insulin administration in the initial 5–7 wk. After 8–12 wk of initiating the insulin administration urine ACE2 activity/creatinine ratio fell significantly in STZ-treated mice compared with STZ-treated mice not treated with insulin (Fig. 8B). Urinary albumin/creatinine ratio was lower in STZ-treated mice with insulin than in STZ mice without insulin administration but the difference did not reach statistical significance (Fig. 8C).

**Urinary ACE2 increases ANG II degradation.** Urine from wild-type and ACE2KO mice matched for creatinine concentrations and representing equal amount of total protein (1 mg) were incubated with exogenous ANG II ($10^{-9}$ M) to examine the contribution of urinary ACE2 to the degradation of this peptide.
Urine from WT mice (n = 3) degraded ANG II more effectively than urine obtained from ACE2-deficient mice (n = 3) (Fig. 9A).

Since db/db mice consistently showed markedly elevated levels of urinary ACE2 activity than db/m controls, we sought to determine whether this increase in urinary ACE2 activity in the urines from db/db mice was associated with increased ANG II-degrading capacity. Urines from db/db mice (n = 11) degraded ANG II more rapidly than urines collected from db/m controls (n = 9) by general linear model (GLM) repeated-measures analysis (Fig. 9B). The half-life of ANG II calculated in urines from db/db mice was also significantly lower than in the db/m urines using a nonparametric test and the median (interquartile range) [0.8 (0.5–1.0) vs. 2.5 (1.6–3.8), P < 0.05, respectively]. When MLN-4760 (10⁻⁶ M), a specific ACE2 inhibitor was added to the urine there was no significant difference in the half-lives of ANG II between the urines from db/db (n = 11) and db/m mice (n = 9) [2.2 (0.9–3.16) vs. 5.95 (1.04–16), P < 0.05 (9C)]. Although not significant (P < 0.081), however, the half-life of ANG II in diabetic urine was reduced in the presence of MLN-4760, which suggests additional degradation of the peptide by proteinases other than ACE2. Altogether, these data show that mouse urine contains enzymatically active ACE2 that significantly influences urinary ANG II-degrading capability, which is increased in db/db urines.

Enzymatically active ACE2 protein in mouse urine. In concentrated urines from db/m and db/db mice of 20–24 wk of age two ACE2 immunoreactive protein bands (75 and 110 kDa) were observed (Fig. 10). The relative abundance of the ACE2 protein band at 110 kDa was significantly increased in urine from db/db (n = 16) compared with urine from db/m mice (n = 14) (199 ± 25% of db/m control, P < 0.005). The relative abundance of the 75-kDa band was also higher in db/db than in db/m (259 ± 57% of db/m control, P < 0.005) (Fig. 10).

To examine whether both bands are also present in the kidney, Western blots were performed in kidneys from a group of 10- to 11-wk-old db/m and db/db mice (n = 5 in each group). As shown in older mice (Fig. 10), urines from these younger db/m and db/db mice also showed an ACE2 immunoreactive band at around 100–110 kDa and also a band at 75 kDa, which was variably seen (Fig. 11A).

Unlike in the urines, in isolated kidney cortex plasma membranes from these younger db/m and db/db mice, only the ~100- to 110-kDa ACE2-immunoreactive band was detectable (Fig. 11). Moreover, in additional studies in whole cell lysates only the 100- to 110-kDa band was found as well (Fig. 11). ACE2/GAPDH ratio for whole lysates and ACE2/B-actin ratio for membrane fractions were both significantly higher in db/db mice compared with db/m (302 ± 12% and 222 ± 25% of the db/m controls, respectively, P < 0.005). ACE2 activity measured in whole kidney cortex lysates from db/db mice was also significantly higher than in db/m (129.8 ± 9.8 vs. 50.3 ± 1.2 RFU·µg protein⁻¹·h⁻¹, respectively, P < 0.001).

Consistent with the Western blot results of the db/m and db/db mouse urines, in concentrated urines from a control wild-type mice (ace2+/+, C57BL/6 genetic background) Western blotting using a specific ACE2 antibody showed two main bands at ~100–110 kDa and 75 kDa whereas in ACE2 knockout mice (ace2−/−, neither band was found (Fig. 12). In the same concentrated urines, there was abundant ACE2 enzymatic activity detectable in WT but not in the ACE2KO (111.5 vs. ~6.0 RFU·µL⁻¹·h⁻¹, respectively).

DISCUSSION

ACE2 is abundantly expressed in the kidney, mainly in tubular epithelial cells and to a lesser extent in glomeruli and the renal vasculature (47, 48). It is believed that the levels of ACE2 activity in the various nephron segments govern the local levels of ANG II in concert with the level of ACE and other enzymes involved in the formation and degradation of ANG II (1, 7, 8, 20, 30). The formation of ANG-(1–7), a peptide that opposes and counterbalances actions of ANG II (4), is also largely dependent on cleavage of ANG II by ACE2 (28, 37, 42, 46).
Activity was not significantly different between the two groups (Fig. 5). This is in keeping with the high molecular weight of soluble ACE2, which renders it nonfilterable. Moreover, rACE2 administration to db/db mice also failed to increase urinary ACE2 activity despite a large increase in serum ACE2 activity. This shows that increased serum ACE2 activity in diabetic mice cannot account for the elevated urinary ACE2 activity. Accordingly, the source of increased urine ACE2 must be the kidney itself since ACE2, when infused, does not appear in the urine.

We also examined the effect of RAS inhibition on urinary ACE2 activity. Urinary ACE2 in db/m and db/db mice was unchanged by either chronic angiotensin type 1 receptor blocker (telmisartan, 2 mg·kg⁻¹·day⁻¹) or ACE inhibitor (captopril, 60 mg·kg⁻¹·day⁻¹) administration, and the large differences between db/m and db/db mice were not affected. One could speculate that the inability of telmisartan and captopril to reduce urinary ACE2 may be beneficial in terms of renal effects of these RAS blockers by not interfering with ACE2-driven ANG II degradation. In addition, the lack of effect would be advantageous for the potential use of urinary ACE2 as biomarker of kidney disease since most patients are currently receiving RAS blockers.

The potential effect of changes in dietary sodium intake on urinary ACE2 protein was also examined. Changing from a normal to a very high-salt diet resulted in a sharp increase in urinary ACE2 activity. This effect was noted both in diabetic and nondiabetic mice. Conversely, a low-salt diet resulted in a fall in the level of urinary ACE2 activity in both diabetic and nondiabetic mice. An increase in ACE2 with a high-salt diet would reduce ANG II levels and therefore promote natriuresis whereas a decrease in ACE2 would increase it and promote sodium retention. The observed changes in urinary ACE2 activity, however, did not follow the predicted changes in plasma ANG II levels (low in a high-salt diet and high in a low-salt diet) and therefore require other explanations. One possibility is that changes in urinary flow imposed by the extreme changes in dietary sodium intake in these experiments may have overruled the effects of these RAS blockers by not interfering with ACE2-driven ANG II degradation. In addition, the lack of effect could speculate that the inability of telmisartan and captopril to reduce urinary ACE2 may be beneficial in terms of renal effects of these RAS blockers by not interfering with ACE2-driven ANG II degradation. In addition, the lack of effect could be advantageous for the potential use of urinary ACE2 as biomarker of kidney disease since most patients are currently receiving RAS blockers.

The mechanism for increased urinary excretion of ACE2 in diabetic kidney disease, however, was not examined in these previous studies. Here we report that in urines from diabetic db/db mice and STZ-treated mice the levels of ACE2 activity are markedly increased compared with nondiabetic controls. This increase was observed in db/db mice already at 7 wk of age, before the development of albuminuria, and increased progressively over time as albuminuria increased (Fig. 1). Moreover, serum ACE2 activity in serum from diabetic mice was also increased, suggesting the possibility of a generalized compensatory mechanism that helps metabolize ANG II more efficiently in these models of diabetic kidney disease. To examine whether the increase in urinary ACE2 activity was of circulatory origin we infused recombinant ACE2 to control and diabetic mice. When db/m mice were infused with soluble recombinant ACE2, a marked increase in circulating (serum) ACE2 activity was observed but there was no effect whatsoever on urinary ACE2 activity (Fig. 5). This is in keeping with the high molecular weight of soluble ACE2, which renders it nonfilterable. Moreover, rACE2 administration to db/db mice also failed to increase urinary ACE2 activity despite a large increase in serum ACE2 activity. This shows that increased serum ACE2 activity in diabetic mice cannot account for the elevated urinary ACE2 activity. Accordingly, the source of increased urine ACE2 must be the kidney itself since ACE2, when infused, does not appear in the urine.

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Alterations in ACE2 have been recognized both in animal models of diabetic kidney disease and humans with diabetic nephropathy (18, 21, 27, 36, 43, 47, 48). More recently, we and others have proposed that the level of ACE2 activity in the urine could potentially serve as a biomarker of diabetic kidney disease (2, 41, 44). Mizuiri et al. (17) recently reported that humans with chronic kidney disease have elevated urinary levels of ACE2 protein compared with control subjects (17). Of note, diabetic patients had higher urinary ACE2 levels than nondiabetics (17). Burns’ group (44) likewise has reported increased levels of urinary ACE2 activity in renal transplant recipients with diabetes compared with nondiabetics.

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affected urinary ACE2 mechanical shedding. According with this possibility, urinary ACE2 activity had a highly significant positive correlation with urine flow (Fig. 2). The high urine flow associated with hyperglycemia in db/db mice could therefore contribute to the observed high levels of urine ACE2 protein. High levels of urinary ACE2 were also seen in a different model of diabetic kidney disease, the STZ-treated mice. In this model, insulin lowered urinary ACE2 activity but only after several weeks of continuous administration which is consistent with a recent study showing that administration of an insulin sensitizer, rosiglitazone, to db/db mice normalized hyperglycemia and decreased urinary ACE2 (2). An improvement in glycemic control and the attendant reduction in osmotic diuresis and urine flow does not seem therefore the only explanation for the lowering effect of insulin on urinary ACE2 which was observed only after several weeks of its administration.

ACE2 is primarily localized to the apical membrane of proximal tubular cells (48) but is also present in parietal and visceral epithelial glomerular cells (48). In previous studies (43, 47) it was found that ACE2 is increased in kidney cortex but decreased by immunostaining in glomeruli from young db/db mice (8 wk of age). In other models of diabetic kidney disease (Akita and in STZ-treated mice) kidney ACE2 protein or activity were also reported to be elevated in kidney cortex (33, 35, 40, 43). In the present study, we measured ACE2 activity in both glomerular and tubular kidney isolates from older db/db mice (32 wk of age) and found that tubular ACE2 activity was increased compared with db/m controls. Moreover, there was a positive correlation between tubular and urinary ACE2 but not between glomerular ACE2 activity and urinary ACE2 activity. These findings all together strongly suggest that the primary source of elevated urinary ACE2 in diabetic kidney disease is of tubular origin, the site of the nephron where this enzyme is more abundantly expressed. We found, moreover, that ACE2 protein was increased in kidney cortex membrane fractions from the db/db mice compared with db/m mice (Fig. 11). Therefore, the elevated levels of urinary ACE2 are likely a reflection of increased tubular ACE2 content in the apical plasma membrane. Further evidence for the notion

![Fig. 9. Degradation of exogenous ANG II by urine from different groups. A: urines from C57BL/6 wild-type mice (WT, n = 3) degraded ANG II more efficiently than those obtained from ACE2 deficient mice on the same genetic background (ACE2KO, n = 3). ***P < 0.01 WT vs. ACE2KO as determined by repeated-measures GLM analysis. B: urines from db/db mice (n = 11) degraded ANG II more efficiently than those collected from db/m (n = 9) *P < 0.05, db/db vs. db/m as determined by repeated-measures GLM analysis. C: degradation of ANG II in the urines from the db/db (n = 11) and db/m mice (n = 9) was not significantly different when a specific ACE2 inhibitor, MLN-4760 (10^-6 M), was added to the urine.](http://ajprenal.physiology.org/.../10.1152/ajprenal.00600.2012)
decreased urinary ACE2 in fetal renal programmed hypertrophy (29).

Apical ACE2 protein could be shed from the membrane of tubular epithelial cells, and therefore an increase in urinary ACE2 activity in diabetic kidneys could reflect a primary increase in tubular ACE2 protein, increased shedding from the apical membrane, or both. Studies in cultured human embryonic kidney cells and airway epithelial cells suggested ACE2 being proteolytically shed from the plasma membrane into the culture media at its carboxy terminus, by a process catalyzed by the enzyme “a disintegrin and metalloproteinase-17” (ADAM17) (10, 11, 13). Of note, renal ADAM17 is upregulated in ANG II-induced kidney injury (14), and de novo expression occurs in human kidney disease, in proximal tubule, podocytes, and mesangial cells (16). While ADAM17 is a recognized sheddase for TNF-α and ACE2 in vitro (26) its role in mediating shedding of soluble ACE2 into the urine from kidney epithelial cells in vivo is not known. It is possible that the increase in urinary ACE2 excretion in db/db mice may be due, in part, to ADAM17-mediated shedding. Because of the increased abundance of ACE2 protein in isolated tubules and in plasma membranes from the db/db mice, however, we think that the increase in urine ACE2 also must reflect augmented tubular ACE2 content. This increase in tubular ACE2 protein and activity would help foster ANG II degradation in a setting where there is RAS overactivity, such as diabetic kidney disease (see below). The increased urinary ACE2 could also be associated with tubular damage in db/db mice; however, renal tubules were reported to be relatively spared from injury in this animal model (31). Thus a correlation between urinary ACE2 and urinary albumin may reflect glomerular damage but the two may not be causally related.

We also assessed urinary ACE2 protein by immunoblotting and found two bands in control and diabetic urine at a molecular mass of 100–110 and 75 kDa. Both these protein bands were absent in urines from ACE2-deficient mice (Fig. 12). The activity associated with the urinary 110-kDa band likely reflects ACE2 activity within the kidney which is where ACE2
resides in the plasma membrane (see Fig. 11). Mouse ACE2 has been shown to have slightly lower molecular mass (110 kDa) than human ACE2 (120 kDa) by two independent groups (19, 24). Therefore the different size of the bigger ACE2 immunoreactive band between human and mouse urinary samples may be a reflection of a difference in molecular weight of human and mouse ACE2 protein. The lower ACE2 band (75 kDa) may be the result of degradation of the soluble ACE2 protein. In support of this hypothesis are the data showing that in isolated kidney lysates the 100- to 110-kDa ACE2 band is only present whereas the 75-kDa ACE2 band appears in the urine with a varying frequency (Fig. 11). Lew et al. (15) reported two bands at approximately 110–115 kDa in healthy human urine samples. Another study showed ACE2 immunoreactive bands at approximately 120 and 90 kDa in healthy subjects and 120 and 75 kDa in patients with chronic kidney disease (17).

The ability of urinary ACE2 to metabolize exogenous ANG II was studied in control and diabetic mice. In a previous study, Lew et al. (15) could not ascertain a significant processing of angiotensin peptides by human urines. For their liquid chromatography-based angiotensin degradation studies, these authors employed amounts of ANG II substrate several thousand times higher (8 × 10^{-5} μM) than those normally present in the native urine, because of the relatively low sensitivity of this assay (15). We used ANG II, at much lower concentration (10^{-9} M) empirically arrived at by a careful titration, and this approach revealed ANG II degradation by mouse urine as previously reported in sheep urine (30). We, moreover, found that ANG II degradation is increased in urine from diabetic mice and that this effect was largely ACE2-dependent because the differences between db/h and db/db urine degrading capacity were not significant in the presence of MLN-4760, a specific ACE2 inhibitor. It is important to acknowledge, however, that there was a component of ANG II degradation that was clearly ACE2-independent. Other peptidases, such as nephrilysin, can also metabolize ANG II in the urine (30). Interestingly, in female diabetic rats Yamaleyeva et al. (45) have observed a marked reduction of kidney nephrilysin activity, and it is possible that urinary nephrilysin may explain why there is rapid degradation of ANG II in the first hour in urine of db/db and db/m mice that is apparently not blocked by the ACE2 inhibitor, MLN-4760.

Using micropuncture studies, ANG II levels in tubular fluid and urine have been reported to be severalfold higher than in plasma (20). Interestingly, we found that ACE2 activity in the urine is on average eightfold higher compared with serum. This would be consistent with the hypothesis that the increased ACE2 in urines from diabetic animals reflects kidney ANG II overactivity. That is, higher levels of ANG II within the kidney may require higher levels of ACE2 activity for its metabolism and this is accomplished by an increase in tubular plasma membrane ACE2 protein and therefore ACE2 activity. Possibly, the increase in ACE2 protein is compensatory to limit the accumulation of ANG II in diabetic kidneys. In any case, ANG II degradation in the native urine by ACE2 and other enzymes is of key importance in the interpretation of urinary peptide concentrations. This ongoing degradation renders the measurements of endogenous ANG II unreliable in terms of reflecting concentration of this peptide in the kidney prior to enzymatic degradation. Therefore, in this sense, urinary ACE2 could be used as a potential biomarker of RAS overactivity in diabetic kidney disease. This information could be valuable in providing guidance as to the timing of therapeutic intervention to block the renin-angiotensin system.

In summary, urinary ACE2 activity is increased in diabetic mice and the source of this increase is of tubular rather than glomerular origin. Salt intake, urine flow, and insulin can all affect urinary ACE2 activity. The activity of ACE2 in the urine significantly influences ANG II degrading capacity and probably reflects protein shed from the tubular plasma membrane. Overall, these findings suggest that increased urinary ACE2 in diabetic kidney disease could be a potential biomarker of increased metabolism of ANG II as a result of RAS overactivity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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