CLINICAL STUDY

High urinary ACE2 concentrations are associated with severity of glucose intolerance and microalbuminuria

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Abstract

Objective: Angiotensin-converting enzyme 2 (ACE2) plays an important role in glucose metabolism and renal function. However, the relationship between ACE2 and hyperglycemia or microalbuminuria has not been established in humans. We investigated whether urinary ACE2 levels are associated with abnormal glucose homeostasis and urinary albumin excretion.

Methods: We developed an ELISA for quantifying ACE2 in urine. The ELISA was used to measure urinary ACE2 levels in 621 subjects with: normal glucose tolerance (NGT; $n=77$); impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) ($n=132$); and type 2 diabetes mellitus (T2DM, $n=412$). Insulin resistance was assessed by homeostasis model assessment for insulin resistance (HOMA-IR) index and urinary albumin excretion by urine albumin-to-creatinine ratio (ACR). Other biochemical and anthropometric parameters were measured.

Results: Urinary ACE2 levels were significantly higher in insulin-resistant subjects with IFG, IGT, and T2DM than in the NGT group ($P<0.001$). Urinary ACE2 concentrations appeared to correlate with HOMA-IR, fasting blood glucose, triglyceride, high-sensitivity C-reactive protein, serum creatinine, urinary ACR, and systolic blood pressure (all $P<0.05$). After adjustment for impaired renal function and other metabolic parameters, urinary ACE2 concentration was still associated with a higher risk for T2DM (OR 1.80, 95% CI 1.05–3.08, $P=0.02$). In addition, urinary ACE2 levels were highly predictive of microalbuminuria after adjusting for clinical risk factors (OR 2.68, 95% CI 1.55–4.64, $P<0.001$). **Conclusion:** Our data suggest that the urinary ACE2 level is closely associated with T2DM and is an independent risk factor for microalbuminuria.

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Introduction

The mechanisms involved in the development of type 2 diabetes mellitus (T2DM) and its complications are complex, with a long list of potential derangements in different pathways (1, 2). Both clinical trials and animal models of T2DM have shown that the renin–angiotensin system (RAS) contributes to the development of T2DM and its complications (3). Of clinical relevance, blockade of the RAS prevents new-onset T2DM and reduces the risk of T2DM and its complications (4, 5).

Angiotensin-converting enzyme (ACE) has long been recognized as the key enzyme within the RAS, as it is involved in cleaving angiotensin (Ang) I to form Ang II, which is the main active peptide within the system. ACE2, a first homolog of ACE, is a monocarboxypeptidase that preferentially removes carboxy-terminal amino acids from various substrates, including Ang II, Ang I, and apelin (6, 7, 8, 9). ACE2 cleaves Ang II to

form Ang-(1–7) (10). Alterations in glucose tolerance and reduced first-phase insulin secretion have been described in ACE2-deficient mice, suggesting a potential role of ACE2 in the development of T2DM (11).

Microalbuminuria is an early marker of diabetic nephropathy and an independent risk factor for cardiovascular disease (12, 13). Previous data have demonstrated that microalbuminuria is associated with insulin resistance in type 2 diabetic patients as well as in nondiabetic individuals (14). Thus, the early identification and treatment of patients at increased risk for microalbuminuria may be important for preventing renal and cardiovascular diseases associated with T2DM. A number of studies have reported a relationship between RAS and proteinuria (15, 16). In the kidney, ACE2 is localized in the proximal tubules and glomerulus (17). Previously, it was reported that renal expression of ACE2 is downregulated in T2DM (18, 19), whereas others showed that ACE2 expression and ACE2

activity were increased in animal models of diabetes (20, 21). Additionally, a positive relationship between urinary mRNA expression of ACE2 and the degree of proteinuria in human type 2 diabetic nephropathy has been reportedly found (22). Soluble ACE2, as well as ACE, can be detected in urine (23). A recent study identified a significant difference in urinary ACE2 levels between subjects with chronic kidney disease (CKD) and healthy subjects (24). Accordingly, ACE2 is important as a potential participant in the development of both T2DM and nephropathy (25). However, a relationship between urinary concentrations of ACE2 and glycemia or microalbuminuria has not been established.

In this study, we investigated the relationships of urinary ACE2 concentrations with glucose metabolism and urinary albumin excretion status. To further evaluate the role of urinary ACE2, we assessed the relationships between urinary ACE2 levels and various metabolic parameters.

Materials and methods

Participants

Between September 2007 and September 2008, subjects aged 21–80 years with a history of hyperglycemia (fasting plasma glucose ≥ 5.5 mmol/ml) or T2DM were enrolled in the Seoul Metro-City Diabetes Prevention Program (SMC-DPP) (26). The subjects were recruited from five public health centers. After exclusion of subjects taking antiobesity medications or corticosteroids and those with histories of diabetic ketoacidosis, symptomatic heart failure, and renal or hepatic dysfunction, assignment to one of the groups was performed using a 75 g oral glucose tolerance test according to the diagnostic criteria of the American Diabetes Association (27). We recruited 621 subjects with: normal glucose tolerance (NGT); impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or IFG+IGT; or T2DM who had adequate, stable renal function (serum creatinine (Cr) <2.0 mg/dl). All participants provided written informed consent. This study was approved by the Institutional Review Board at Kangbuk Samsung Hospital.

Anthropometric and laboratory measurements

BMI (kg/m^2) was calculated as body weight in kilograms divided by height in meters squared. Blood pressures were measured using a sphygmomanometer (Welch Allyn, Inc., Vital Signs Monitor 300 series, Skaneateles Falls, NY, USA) between 0800 and 1000 h after at least 5 min of rest, according to the Hypertension Detection and Follow-up Program protocol (28). Trained nurses measured seated blood pressure. When the SBP or DBP exceeded 140 or

90 mmHg, it was remeasured after a 5-min rest, and the results averaged.

Blood samples were collected following an overnight fast. The subjects also provided the first morning specimen of urine. Aliquots of both plasma and urine were stored at -80°C until assayed. Height, weight, and waist circumferences were measured. Plasma glucose concentrations were determined using a Beckman glucose analyzer II (Beckman Instruments, Fullerton, CA, USA). HbA1c was measured using HPLC (Variant II, Bio-Rad Laboratories). The assay coefficient of variability (CV) for glucose was <1.5 and $<3.0\%$ for HbA1c. Serum insulin levels were measured using an immunoradiometric assay (Bio-source, Nivells, Belgium) following the manufacturer's recommendations. Plasma lipids, including total cholesterol, triglyceride, HDL-C, and LDL-C, were measured by enzymatic colorimetric assay (Siemens, Tarrytown, NY, USA). Serum high-sensitivity C-reactive protein (hsCRP) levels were measured by nephelometric assay using a BNII nephelometer (Dade Behring, Deerfield, IL, USA), and the detection limit was 0.175 mg/l with a sample dilution of 1:20. Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as described previously (29). Serum creatinine was measured using the timed-end point method (Unicel Dx C 800, Beckman and Coulter, Krefeld, Germany).

Urinary albumin-to-creatinine ratio (ACR) was calculated using consecutive morning and daytime urine specimens by an immunonephelometric method using the DCA 2000 albumin/creatinine ratio urinalyzer (Bayer Corp.). The intra-assay and interassay CV were reported as being within the range 2 and 8% for ACR. Microalbuminuria was defined as an ACR of 30–300 $\mu\text{g}/\text{mg}$ creatinine. Glomerular filtration rate was estimated by the Modification of Diet in Renal Disease (MDRD) Study Group formula (estimated glomerular filtration rate, eGFR). In accordance with the National Kidney Foundation Disease Outcomes Quality Initiative classification system, CKD was defined on the basis of eGFR and categorized into three groups defined by the cutoff points. Therefore, subjects in the stage 1–2 CKD group had eGFRs ≥ 60 ml/min per 1.73 m^2 , the stage 3 CKD group had eGFRs <60 to ≥ 30 ml/min per 1.73 m^2 , and the stage 4–5 CKD group had eGFRs <30 ml/min per 1.73 m^2 (30). Subjects with an eGFR ≥ 90 ml/min per 1.73 m^2 without proteinuria or history of kidney disease were placed in stage 0.

Development of human ACE2 ELISA and measurement of urinary ACE2 concentrations

A cDNA sequence encoding the original signal peptide sequence and the mature peptide of human ACE2, excluding the transmembrane domain, and a short stretch of cytoplasmic tail, was amplified with the

following primer set: i) forward primer 5'-cctagctagcgg-gacgatgtcaagctcttc-3' and ii) reverse primer 5'-gccgctcgagggtggttaggaggtccaag-3'. The sequence was then digested with NheI and XhoI and cloned into both pAGCF (AdipoGen, Inccheon, Korea). A FLAG tag was incorporated at the COOH terminus of the human ACE2 peptide. The tagged protein was expressed in a human embryonic kidney cell line, HEK-293, and purified from conditioned media through an anti-FLAG sepharose column. Polyclonal antibody (PAb) was then produced by immunization with recombinant FLAG-tagged human ACE2 according to a general protocol. Immunoglobulin fractions were prepared from serum and then biotinylated. FLAG-tagged ACE2 was used as the ELISA standard at a variety of dilutions. A sandwich ELISA format was designed using a pair of PAb and biotinylated PAb. One hundred microliters of human urine in 1:2 dilutions were applied to each well, which had been coated with 5 µg/ml of a human ACE2-specific PAb and incubated at 37 °C for 1 h, followed by washing three times with PBS with 0.05% Tween 20 (PBST). One hundred microliters of the biotinylated PAb at 5 mg/ml were added per well. The secondary antibody reaction was performed at 37 °C for 1 h, followed by washing three times with PBST. A colorimetric reaction was conducted for 20 min using HRP-conjugated streptavidin (Zymed, South San Francisco, CA, USA) diluted 1:1000 in PBS and 2,2'-azino-bis(2-ethylbenzothiazoline-6-sulfonic acid) (Pierce, Rockford, IL, USA) as the substrate. The optical density was measured at 450 nm; its sensitivity was 293 pg/ml. While the degree of precision of the ELISA system in terms of the intra-assay CV was between 6.1 and 9.9% (Supplementary Table 1, see section on supplementary data given at the end of this article), the inter-assay CV were between 5.4 and 10.8% (Supplementary Table 2). Spike recovery (Supplementary Table 3) and linearity (Supplementary Table 4) were in the ranges of 90–100 and 89–107% respectively. Specificity was determined such that it would not cross-react with human ACE1, mouse ACE2, human adiponectin, human leptin, human resistin, human visfatin, human clusterin, human retinal binding protein 4, human resistin-like molecule- α (RELM- α), human IL23, human angiotensin 1, human angiotensin 2, human fatty acid binding protein 4, human angiotensin-like protein 6 (ANGPTL6), human plasminogen activator inhibitor-1 (PAI-1), human vaspin, or mouse RELM- β (Supplementary Table 5).

Statistical analysis

Analyses were performed using SPSS for Windows software (version 17.0; SPSS). We used ANOVA, Wilcoxon rank sum tests, and Kruskal–Wallis tests for comparisons of continuous variables and χ^2 tests for comparison of categorical variables. Bivariate correlation analyses between urinary ACE2 and the

metabolic parameters were performed using Pearson's correlation analysis. Multivariate logistic regression analysis was performed to investigate associations between T2DM or microalbuminuria with urinary ACE2 levels. *P* values <0.05 were considered significant.

Results

Baseline characteristics of the study participants are shown in Table 1. The mean age ($\pm$ s.d.) was 59.8 ($\pm$ 12.8) years and 51.4% of the participants were male. The duration of diabetes in T2DM subjects was 3.1 $\pm$ 5.2 years. With increasing impairment of glucose metabolism, we detected expected group differences in age, waist circumference, fasting blood glucose, HbA1c, total cholesterol, triglyceride, HDL-C, fasting insulin, insulin sensitivity (HOMA-IR), hsCRP, and blood pressure values. Subjects with T2DM exhibited evidence of impaired renal function, as indicated by higher serum Cr, lower eGFR, and urinary ACR values. There were significant differences in urinary ACE2 concentrations between subjects with NGT, IFG, or IGT and T2DM (all *P* < 0.05). Among the subjects, 41.7, 40.4, 15.5, and 20.4% had stage 0, 1, 2, and 3 for CKD respectively. Urinary ACE2 levels were increased according to the stage of CKD. The median (interquartile range) for urinary ACE2 was 2.19 (1.30–4.33) for CKD stage 0, 2.58 (1.52–4.14) for CKD stage 1, 3.78 (2.12–6.55) for CKD stage 2, and 6.59 (3.54, 9.41) for CKD stage 3 respectively (*P* < 0.01). After adjusting for age, sex, and BMI, urinary ACE2 concentrations were correlated with fasting blood glucose, triglyceride, HOMA-IR, hsCRP, systolic blood pressure, serum Cr, and urinary ACR levels (Table 2, all *P* < 0.05).

Urinary ACE2 levels were grouped into tertiles to simplify interpretation of the results of subsequent analyses. With respect to metabolic parameters, subjects in the higher ACE2 tertiles exhibited higher values for age, fasting blood glucose, HbA1c, triglyceride, HOMA-IR, hsCRP, systolic blood pressure, serum Cr, and urinary ACR (all *P* < 0.05) than those in the lower tertile (Table 3).

As shown in Table 4, increased urinary ACE2 concentrations were associated with an increased risk of hyperglycemia. Subjects with a higher level of urinary ACE2 had a higher risk of T2DM after adjusting for age, sex, BMI, and several metabolic parameters (OR 1.80, 95% CI 1.05–3.08, *P* = 0.033). The corresponding OR for both impaired glucose regulation (IFG or IGT) and T2DM was 1.88 (95% CI 1.01–3.47, *P* = 0.045).

Multivariate linear regression analysis also showed that urinary ACE2 concentrations were associated with a high risk of microalbuminuria (OR 2.68, 95% CI 1.55–4.64, *P* < 0.001) even after controlling for clinical risk factors (Table 5).

Table 1 Baseline characteristics of the study sample. Data are summarized as mean $\pm$ s.d., median (interquartile range) for non-normal distribution, or *n* (%). HbA1c is expressed as % and mmol/l.

	NGT (<i>n</i> =77)	IFG or IGT (<i>n</i> =132)	T2DM (<i>n</i> =412)	<i>P</i> value
Age (years)	41.3 $\pm$ 10.2	58.8 $\pm$ 12.4	63.5 $\pm$ 10.1	<0.001
Sex (M/F)	60/17	60/72	199/213	<0.001
BMI (kg/m ²)	22.7 $\pm$ 3.3	24.6 $\pm$ 3.3	25.5 $\pm$ 11.4	0.056
Waist circumference (cm)	78.4 $\pm$ 9.9	84.4 $\pm$ 11.5	88.1 $\pm$ 15.7	<0.001
Fasting blood glucose (mmol/l)	5.30 $\pm$ 0.60	6.31 $\pm$ 0.59	8.39 $\pm$ 2.13	<0.001
HbA1c (%; mmol/mol)	5.39 $\pm$ 0.12 (35.38 $\pm$ 4.56)	5.79 $\pm$ 0.36 (39.74 $\pm$ 3.91)	7.35 $\pm$ 1.33 (56.80 $\pm$ 14.49)	<0.001
Total cholesterol (mmol/l)	4.66 $\pm$ 0.79	0.51 $\pm$ 0.94	4.87 $\pm$ 0.98	0.007
Triglycerides (mmol/l)	0.90 (0.55–1.48)	1.36 (0.98–1.81)	1.52 (1.08–2.22)	<0.001
HDL-C (mmol/l)	1.38 $\pm$ 0.31	1.37 $\pm$ 0.32	1.28 $\pm$ 0.34	0.005
LDL-C (mmol/l)	2.84 $\pm$ 0.78	2.90 $\pm$ 0.83	2.71 $\pm$ 0.85	0.064
Fasting insulin (pmol/l)	39.6 (25.2–53.5)	63.3 (51.1–82.0)	72.9 (57.2–99.9)	<0.001
HOMA-IR (arbitrary unit)	2.07 (1.53–2.85)	2.52 (2.02–3.48)	3.90 (2.92–5.30)	<0.001
hsCRP (mg/l)	0.06 (0.04–0.10)	0.06 (0.03–0.13)	0.09 (0.04–0.22)	<0.001
Taking antihypertensive drugs, <i>n</i> (%)	2 (2.6)	49 (37.1)	245 (59.5)	<0.001
Systolic blood pressure (mmHg)	114.1 $\pm$ 10.8	128.9 $\pm$ 9.4	133.6 $\pm$ 18.7	<0.001
Diastolic blood pressure (mmHg)	70.9 $\pm$ 8.3	83.7 $\pm$ 2.8	83.4 $\pm$ 12.0	<0.001
Urinary ACR (mg/g)	6.0 (5.0–10.8)	11.5 (6.9–32.0)	23.3 (9.8–63.1)	<0.001
Serum creatinine concentration (mg/dl)	0.85 $\pm$ 0.19	0.74 $\pm$ 0.20	0.78 $\pm$ 0.20	0.001
eGFR (ml/min per 1.73 m ²)	100.0 $\pm$ 22.4	92.5 $\pm$ 29.5	82.9 $\pm$ 26.9	<0.001
Urinary ACE2 (ng/ml)	2.18 (1.38–3.70)	2.44 (1.33–3.96)	3.00 (1.61–4.97)	0.002
Current smoking (%)	2 (2.6)	13 (9.8)	59 (14.3)	0.010
Current alcohol intake (%)	5 (6.5)	52 (39.4)	11.9 (28.9)	<0.001

NGT, normal glucose tolerance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; T2DM, type 2 diabetes mellitus; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; ACR, albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate.

Discussion

This study demonstrated that urinary ACE2 levels appear to be elevated in subjects with T2DM and are associated with various metabolic parameters. We also found that urinary ACE2 levels are associated with a higher risk of microalbuminuria, independent of various confounding factors. The results of this study imply that urinary ACE2 concentration may be a valuable marker for both glucose intolerance and microalbuminuria.

Previous studies suggest that ACE2 may play a pivotal role in T2DM. Ang II can delay insulin secretion and reduce blood flow in the islets of Langerhans in rodents. Consistent with this effect of Ang II, blockade of the RAS with either ACE inhibitors or Ang II receptor blockers increases islet blood flow (31). These findings are particularly relevant given clinical evidence that RAS blockade may be associated with reduced incidence of new-onset T2DM (32). ACE2 is elevated in T2DM and may be involved in a compensatory mechanism opposing the ACE/Ang II/AT1 receptor axis, leading to improved glucose tolerance in animal models (3). We therefore hypothesized that urinary ACE2 concentrations are closely related to glucose intolerance and metabolic parameters.

We developed an ELISA for the measurement of human urinary ACE2 concentrations. Takahashi *et al.* (33) employed an immunoprecipitation technology for

Table 2 Correlations between urinary ACE2 and various metabolic parameters.

	Urinary ACE2 (ng/ml) ^a			
	Unadjusted		Age and sex adjusted	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Age	0.095	0.018	–	–
BMI	–0.027	0.510	–	–
Waist circumference	0.067	0.102	–	–
Fasting glucose ^a	0.184	<0.001	0.173	<0.001
Total cholesterol	–0.028	0.489	–0.021	0.627
Triglycerides ^a	0.145	<0.001	0.150	0.001
HDL-C	–0.089	0.028	–0.077	0.080
LDL-C	–0.063	0.126	–0.057	0.198
Fasting insulin ^a	0.045	0.262	0.078	0.077
HOMA-IR ^a	0.113	0.008	0.137	0.002
hsCRP ^a	0.126	0.002	0.141	0.001
Systolic blood pressure	0.117	0.004	0.129	0.003
Diastolic blood pressure	0.034	0.398	0.039	0.380
Urinary ACR ^a	0.319	<0.001	0.312	<0.001
Serum creatinine concentration	0.137	0.001	0.099	0.024
eGFR	–0.083	0.044	–0.049	0.271

HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; ACR, albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate.

^a.37wLogarithmic transformation was performed because of a skewed distribution.

Table 3 Metabolic risk factors according to urinary ACE2 tertile. Data are summarized as mean $\pm$ s.d., median (interquartile range) for non-normal distribution.

Characteristics	I (n=207) ≤ 1.92 ng/ml	II (n=209) 1.93–3.76 ng/ml	III (n=205) ≥ 3.77 ng/ml	P value
Age (years)	58.9 $\pm$ 12.3	58.7 $\pm$ 13.1	61.7 $\pm$ 12.9	0.030
Sex (M/F)	92/115	112/97	115/90	0.045
BMI (kg/m ²)	25.7 $\pm$ 15.7	24.6 $\pm$ 3.3	24.6 $\pm$ 3.4	0.372
Waist circumference (cm)	83.5 $\pm$ 16.3	85.4 $\pm$ 12.4	85.4 $\pm$ 14.3	0.330
Fasting blood glucose (mmol/l)	7.28 $\pm$ 1.73	7.39 $\pm$ 2.02	8.02 $\pm$ 2.51	0.001
HbA1c (%)	6.65 $\pm$ 1.24	6.77 $\pm$ 1.34	7.17 $\pm$ 1.50	<0.001
Total cholesterol (mmol/l)	4.99 $\pm$ 0.93	4.86 $\pm$ 0.94	4.83 $\pm$ 1.01	0.212
Triglycerides (mmol/l)	1.29 (0.94–1.79)	1.47 (0.96–2.24)	1.46 (1.08–2.16)	0.008
HDL-C (mmol/l)	1.35 $\pm$ 0.32	1.30 $\pm$ 0.33	1.28 $\pm$ 0.34	0.084
LDL-C (mmol/l)	2.85 $\pm$ 0.82	2.78 $\pm$ 0.86	2.67 $\pm$ 0.85	0.129
Fasting insulin (pmol/l)	66.4 (50.3–64.7)	67.9 (50.9–94.6)	70.0 (52.2–88.3)	0.432
HOMA-IR (arbitrary unit)	3.26 (2.41–4.46)	3.64 (2.45–5.08)	3.67 (2.72–4.72)	0.048
hsCRP (mg/l)	0.07 (0.03–0.17)	0.07 (0.03–0.17)	0.10 (0.05–0.21)	0.003
Taking antihypertensive drugs, n (%)	83 (40.1)	95 (45.5)	118 (57.6)	0.001
ACEi/ARB (%)	30 (14.5)	34 (16.3)	38 (18.5)	0.540
Systolic blood pressure (mmHg)	129.0 $\pm$ 18.8	128.3 $\pm$ 18.2	133.3 $\pm$ 20.0	0.017
Diastolic blood pressure (mmHg)	82.2 $\pm$ 12.3	81.1 $\pm$ 2.6	82.6 $\pm$ 12.6	0.441
Urinary ACR (mg/g)	11.3 (6.6–31.5)	14.2 (7.1–43.8)	24.8 (9.0–97.1)	<0.001
Serum creatinine concentration (mg/dl)	0.75 $\pm$ 0.19	0.78 $\pm$ 0.19	0.82 $\pm$ 0.23	0.005
eGFR (ml/min per 1.73 m ²)	88.8 $\pm$ 26.6	88.3 $\pm$ 26.5	84.1 $\pm$ 29.8	0.179

HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; ACEi/ARB angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker; ACR, albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate.

detection of serum ACE2 after removing nucleic acids with the use of an antihuman ACE2 antibody. In parallel with the use of our urinary ACE2 immunoassay, Mizuiri *et al.* (24) concentrated human sera after removing serum IgG and was able to detect serum ACE2. Regarding the measurement of human ACE2, Dilauro *et al.* (34) utilized a fluorescence dye-based enzymatic assay that was distinguished from ACE. Recently, Xiao *et al.* (35) described the presence of urinary ACE2 using an effective western blot analysis in comparison with the current immunoassay and mRNA expression levels, in human urine samples and tissues of a group of diabetic patients with renal transplant. This study showed that both the current ELISA and the western blot were the far more reliable means for measuring the presence of urinary ACE2 than the enzymatic activities in terms of statistics, suggesting that the current ELISA can be more widely and safely applied to measurement of urinary ACE2.

In the current study, urinary ACE2 concentrations were elevated in patients with glucose intolerance and positively related to fasting blood glucose, suggesting that elevated urinary ACE2 might reflect the severity of glucose intolerance. This is the first study to demonstrate that urinary ACE2 levels might reflect the severity of glucose intolerance. We also found that urinary ACE2 concentrations are correlated with metabolic parameters and the insulin resistance index assessed by HOMA-IR. Recently, Ang-(1–7) binding to the Mas receptor was shown to inhibit Ang II responses (36). Santos *et al.* (37) reported that Mas^{–/–} mice exhibit impaired insulin sensitivity and glucose tolerance, reduced adipose glucose uptake, and impaired glucose transporter 4, which implies that the ACE2/Ang-(1–7)/Mas receptor axis is closely related to the development of insulin resistance. Although the precise mechanisms explaining the role of ACE2 in glucose metabolism are not understood, the link between ACE2 and insulin

Table 4 Risk of type 2 diabetes mellitus with an increase in log(urinary ACE2 (ng/ml)). Values are ORs (95% CI).

Model	Adjustment	Type 2 diabetes mellitus	P value
Model 1	Unadjusted	1.47 (1.37, 3.40)	0.002
Model 2	Adjusted for age, sex, current smoking, and current alcohol intake, based on Model 1	1.92 (1.16, 3.19)	0.011
Model 3	Further adjusted for BMI, total cholesterol, insulin, and hsCRP, based on Model 2	1.85 (1.08, 3.16)	0.024
Model 4	Further adjusted for ACEi/ARB use, based on Model 3	1.80 (1.05, 3.08)	0.033

hsCRP, high-sensitivity C-reactive protein; ACEi/ARB, angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker.

Table 5 Risk of microalbuminuria associated with an increase in log(urinary ACE2 (ng/ml)). Values are ORs (95%).

Model	Adjustment	Microalbuminuria (n=208)	P value
Model 1	Unadjusted	3.35 (2.06, 5.45)	<0.001
Model 2	Adjusted for age, sex, BMI, current smoking, and current alcohol intake, based on Model 1	3.15 (1.90, 5.21)	<0.001
Model 3	Further adjusted for total cholesterol, insulin, and hsCRP, based on Model 2	2.74 (1.60, 4.67)	<0.001
Model 4	Further adjusted for systolic blood pressure, ACEi/ARB use, and eGFR, based on Model 3	2.68 (1.55, 4.64)	<0.001

hsCRP, high-sensitivity C-reactive protein; ACEi/ARB, angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker; eGFR, estimated glomerular filtration rate.

resistance and other metabolic parameters could provide one explanation with respect to glucose metabolism.

Additionally, we showed that ACE2 appears to be positively associated with inflammatory cytokines reflected by hsCRP concentrations. Ang II is well known for its pro-inflammatory action (38), while several studies have demonstrated the ability of ACE2 to inhibit these effects in a disease state (39). Increased inflammation contributes to the development of islet dysfunction and loss of islet morphology (40), which is one potential cause of pancreatic β -cell failure (41). It is possible that ACE2 might have a causal role in systemic inflammation related to the development of T2DM. However, it appears that the relationship between urinary ACE2 and T2DM is consistent, independent of the presence of an inflammatory marker. The mechanism remains unclear, but these results suggest that urinary ACE2 might play a direct role in the severity of glucose intolerance through other mechanisms that do not fully overlap with those of the inflammatory pathways. Further studies are needed to determine whether ACE2 plays a causal role in the regulation of glucose metabolism in humans.

Urinary ACE2 levels are associated with microalbuminuria in our study. Additionally, urinary ACE2 levels are increased according to the stage of CKD. Although this study cannot affirm the cellular origin of urinary ACE2 protein, it could originate at least partly from plasma via glomerular filtration, or it could be derived via excretion from renal cells. A recent study reported increased urinary levels of ACE2 in CKD and diabetic nephropathy, whereas there were no differences in serum ACE2 protein expression (24). However, patients with CKD in their study had significant albuminuria, suggesting that ACE2 may have leaked into the urine across the glomerular barrier. ACE2 is shed at its carboxy-terminus from the plasma membrane in cultured human embryonic kidney cells and airway epithelial cells, a process catalyzed by the enzyme 'a disintegrin and metalloproteinase-17' (ADAM-17) (42, 43), but evidence for shedding ACE2 *in vivo* is limited. Other possibility is that ACE2 protein might derive via shedding from cells along the nephron by ADAM-17 activated by high blood glucose (44), which

might lead to higher urinary ACE2 levels in subjects with CKD. These inverse relationships between urinary expression of ACE2 and renal function imply that urinary ACE2 expression might be a compensatory response of renal tissue to insults. Therefore, urinary ACE2 concentrations might clinically serve as a marker for microalbuminuria and its possible role as a marker for renal impairment needs to be explored further.

There were several limitations to our study. First, due to the cross-sectional nature of our study, we were not able to examine the temporal relationship between urinary ACE2 levels and the severity of glucose intolerance and related metabolic parameters. Long-term follow-up of larger groups is needed to establish the predictive relationship between urinary ACE2 concentrations and metabolic parameters and renal function. Additionally, our study subjects were not drug-naïve, and some subjects were on antihypertensives, glucose-lowering medications, or both, which could influence both urinary ACE2 concentrations and renal excretion of albumin. However, we did not observe significant differences in the urinary ACE2 levels for subjects undergoing ACE inhibitor or Ang II receptor blocker therapy ($P=0.14$, data not shown). Finally, we could not measure the serum levels of ACE2 with the use of the current ELISA assay in our study, likely due to the presence of very low levels of serum ACE2. Evidence for this interpretation was recently provided by Mizuiri *et al.* (24) in that they were able to detect serum ACE2 in western blot analysis only after 20-fold concentration in subjects whose expression patterns remained unchanged in response to CKD.

Nonetheless, our results suggested that urinary ACE2 levels are elevated in subjects with T2DM and are independently associated with a higher risk of microalbuminuria. We also detected significant relationships between urinary ACE2 concentrations and various metabolic and inflammatory markers. Urinary ACE2 concentrations will provide additional information about the role of kidney RAS and may be useful as a marker of glucose intolerance and renal dysfunction among subjects with glucose intolerance. Prospective studies are warranted to elucidate the role of urinary ACE2 measurement.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-12-0782>.

Declaration of interest

J W Park, N Lee, and B-S Youn are employees of AdipoGen, Inc. The other authors have nothing to disclose.

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