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Loss of Angiotensin-Converting Enzyme-2 (*Ace2*) Accelerates Diabetic Kidney Injury

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Diabetic nephropathy is one of the most common causes of end-stage renal failure, but the factors responsible for the development of diabetic nephropathy have not been fully elucidated. We examined the effect of deletion of the angiotensin-converting enzyme 2 (*Ace2*) gene on diabetic kidney injury. *Ace2*$^{-/-}$ mice were crossed with Akita mice (*Ins2*$^{WT/C96Y}$), a model of type 1 diabetes mellitus, and four groups of mice were studied at 3 months of age: *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$, *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$, *Ace2*$^{+/y}$ *Ins2*$^{WT/C96Y}$, and *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$. *Ace2*$^{-/y}$ *Ins2*$^{WT/C96Y}$ mice exhibited a twofold increase in the urinary albumin excretion rate compared with *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice despite similar blood glucose levels. *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice were the only group to exhibit increased mesangial matrix scores and glomerular basement membrane thicknesses compared with *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice, accompanied by increased fibronectin and α-smooth muscle actin immunostaining in the glomeruli of *Ace2*$^{-/y}$ *Ins2*$^{WT/C96Y}$ mice. There were no differences in blood pressure or heart function to account for the exacerbation of kidney injury. Although kidney levels of angiotensin (Ang) II were not increased in the diabetic mice, treatment with an Ang II receptor blocker reduced urinary albumin excretion rate in *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice, suggesting that acceleration of kidney injury in these mice is Ang II-mediated. We conclude that ACE2 plays a protective role in the diabetic kidney, and ACE2 is an important determinant of diabetic nephropathy. ***(Am J Pathol 2007, 171:438–451; DOI: 10.2353/ajpath.2007.060977)***

Activation of the renin-angiotensin system (RAS) and the generation of angiotensin (Ang) II play an important role in the pathogenesis of diabetic nephropathy and blockade of the RAS in both experimental and clinical diabetes mellitus attenuates the development of diabetic kidney injury.[1–3] The recent discovery of a homologue of classical angiotensin-converting enzyme (ACE), angiotensin-converting enzyme 2 (ACE2), has revealed that Ang peptide processing is more complicated than previously thought.[4] Like ACE, ACE2 is membrane-bound, but it is a monocarboxypeptidase that generates Ang (1-9) from the decapeptide Ang I and Ang (1-7) from Ang II.[4] ACE2 serves the dual function of degrading the vasoconstrictor Ang II and producing the vasodilator Ang (1-7).[4] Moreover, Su et al[5] have reported that Ang (1-7) antagonizes Ang II-mediated cell signaling and limits Ang II-induced expression of transforming growth factor-β1. However, the role of ACE2 in mediating diabetic kidney injury has not been fully elucidated.

In diabetic rodent models, early increases in ACE2 mRNA levels, protein expression, and activity have been reported,[6,7] whereas ACE2 mRNA and protein levels have been found to decrease in older streptozotocin-induced diabetic rats.[8] Taken together, these studies suggest that ACE2 may play an early protective role in the development of diabetic nephropathy. To test this hypothesis directly, we used Akita mice (*Ins2*$^{WT/C96Y}$), a model of type I diabetes mellitus, and mice with a dele-

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tion of the *Ace2* gene (*Ace2*$^{-/y}$).[9] The Akita mouse harbors a missense mutation of the insulin 2 gene that leads to misfolding of native insulin in the endoplasmic reticulum of pancreatic β cells, activation of the unfolded protein response, and apoptosis, which eventually renders the mice insulin-deficient.[10] The *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mouse is a preferred murine model for the study of diabetic nephropathy because the mouse develops sustained hyperglycemia, renal hypertrophy, increased urinary albumin excretion rates (AERs), and mesangial matrix expansion, features that are similar to human diabetic nephropathy.[9] We crossed male *Ace2*$^{+/y}$-*Ins2*$^{WT/C96Y}$ mice with female *Ace2*$^{-/-}$*Ins2*$^{WT/WT}$ mice to determine whether the deletion of the *AceII* gene would exacerbate the functional and structural changes of diabetic nephropathy in the Akita mouse. We then treated *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice and *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice with an Ang II type 1 (AT_1) receptor blocker and measured kidney Ang peptide levels to determine whether the effect of deletion of the *AceII* gene in the diabetic mice was Ang II-dependent.

Materials and Methods

Experimental Animals and Protocol

C57BL/6J and diabetic heterozygous Akita (*Ins2*$^{WT/C96Y}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility. *Ace2*$^{-/y}$ mice were outbred on the C57BL/6J background for at least seven generations, and littermate control mice, *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$, were used in the current experiments. To generate the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice, female *Ace2*$^{-/-}$ mice were crossed with male *Ins2*$^{WT/C96Y}$ mice (Figure 1) because the *AceII* gene is on the X chromosome and the Akita mutation (*Ins2*$^{WT/C96Y}$) is a dominant allele. Only male mice were used in the final experiments to simplify the breeding strategy. Throughout the period of study, animals were provided with free access to water and standard 18% protein rodent chow (Harlan Teklad, Madison, WI). *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice and *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice were also treated from weaning until 3 months of age with the ARB, irbesartan (50 mg/kg) by daily gavage (kindly provided by Dr. J. Froehlich, Bristol-Myers Squibb Inc., Montreal, QC, Canada). Twenty-four-hour urine volumes were collected at the end of 3 months and albumin excretion rate (AER) measured. All experiments were conducted in accordance with the guidelines of the University of Toronto Animal Care Committee.

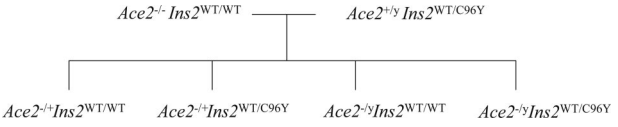

Figure 1. Breeding strategy. Female *Ace2*$^{-/-}$*Ins2*$^{WT/WT}$ mice were bred with male *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice.

Genotyping

DNA was isolated from tail clips using the sodium dodecyl sulfate-potassium acetate method and the mice were genotyped for the *Ins2* and *AceII* genes. The protocol for the Akita genotyping was obtained from The Jackson Laboratory. Briefly, the primers used were 5′-TGCTGATGCCCTGGCCTGCT-3′ and 5′-TGGTCCCACATATGCACATG-3′ (ACGT Corp., Toronto, ON, Canada). The polymerase chain reaction (PCR) cycling conditions were 94°C for 3 minutes, followed by 12 cycles of 94°C for 20 seconds, 64°C for 30 seconds, and 72°C for 35 seconds, then 25 cycles of 94°C for 20 seconds, 58°C for 30 seconds, and 72°C for 35 seconds, and ended with 72°C for 2 minutes. This PCR reaction amplifies a 280-bp DNA fragment from both mutant and wild-type alleles. The PCR products were then incubated at 37°C with FnU4HI restriction enzyme (1 U/0.2 μl; New England Biolabs, Pickering, ON, Canada), which digested wild-type alleles to 140 bp but not mutant alleles, and were resolved on a 2.5% agarose gel.

Blood Glucose Measurements

Body weights and blood glucose levels were obtained monthly. Blood glucose levels were measured monthly between 8:30 AM and 10:30 AM using an Ascensia Breeze glucometer (Bayer Inc., Toronto, ON, Canada). Approximately 3 μl of blood were collected in conscious mice via tail vein puncture. In pilot studies, we measured the blood glucose concentration weekly in diabetic mice for up to 12 weeks. Hyperglycemia was stable and sustained: between 27.3 and 33.1 mmol/L in the *Ace2*$^{+/y}$-*Ins2*$^{WT/C96Y}$ mice and between 30.1 and 33.3 mmol/L in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice.

Urinary AER Measurements

Twenty-four-hour urine collections were obtained from mice before sacrifice by housing them in individual mouse metabolic cages (Nalgene; Nalge Nunc International, Rochester, NY) with free access to water and rodent mash. Urinary albumin concentration was measured using an indirect competitive enzyme-linked immunosorbent assay according to the manufacturer's instructions (Albuwell M; Exocell, Philadelphia, PA).

Histopathology and Electron Microscopy

At 3 months of age, kidneys were harvested for pathological examination. The right kidney was removed before the left, and each was weighed and divided crosswise in three: one section was fixed in 10% neutral buffered formalin (Sigma-Aldrich Co., St. Louis, MO) for 24 hours and then transferred to 90% ethanol for light microscopy and immunohistochemistry, one section was fixed for electron microscopy, and the remaining four sections were snap-frozen for RNA extraction. The formalin-fixed tissue was embedded in paraffin and 2-μm sections were stained with periodic acid-Schiff stain. Slides

Table 1. Primer and Probe Sequences for Real-Time RT-PCR

mRNA	Primer/probe	Sequence
ACE	Forward primer	5′-TGAGAAAAGCACGGAGGTATCC-3′
	Reverse primer	5′-AGAGTTTTGAAAGTTGCTCACATCA-3′
	Probe	5′-FAM-ACCCTGAAATATGGCACCCGGGC-TAMRA-3′
ACE2	Forward primer	5′-GGATACCTACCCTTCCTACATCAGC-3′
	Reverse primer	5′-CTACCCCACATATCACCAAGCA-3′
	Probe	5′-FAM-CCACTGGATGCCTCCCTGCCC-TAMRA-3′
BKB2R	Forward primer	5′-ATGTTCAACGTCACCACACAAGT-3′
	Reverse primer	5′-GGCAGTTGTCCTTCGAAAGG-3′
	Probe	5′-FAM-CTCGGGTCTGCTCTTAACGGGA-TAMRA-3′

were scanned digitally by the Advanced Optical Microscope Facility (Princess Margaret Hospital, Toronto, ON, Canada), and mean cross-sectional areas were calculated using Aperio ImageScope software (Aperio Technologies Inc., Vista, CA). Glomerular volume ($\bar{V}_G$) was calculated from the mean cross-sectional area ($\bar{A}_G$) of 33 glomerular profiles on each animal using the following equation:

$$\bar{V}_G = \beta/k \cdot (\bar{A}_G)^{3/2}$$

where $\beta = 1.38$ is the shape coefficient for spheres (the idealized shape of glomeruli) and $k = 1.1$ is the size distribution coefficient.[11] Glomerular mesangial area was scored from 0 to 4, based on the presence or absence of increased mesangial matrix in each glomerular quadrant, on 34 glomerular profiles from a single coronal kidney section, and the mean was entered as the score for the animal. For electron microscopy, tissue was fixed in buffered 1% glutaraldehyde-4% formaldehyde, postfixed in 1% osmium tetroxide, and embedded in epon-araldite. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1200 EX-II transmission electron microscope (JEOL, Tokyo, Japan). Glomerular basement membrane (GBM) widths were derived using the orthogonal intercept method and the harmonic mean was calculated on 100 widths for each mouse.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were used for all immunohistochemical analysis from mouse kidneys. Heat-induced antigen retrieval was used for all antibodies. Endogenous peroxidase activity was prevented by pretreating all sections with 3% hydrogen peroxide. Primary antibodies and dilutions used were anti-fibronectin (1:1000; BD Transduction, San Diego, CA) and α-smooth muscle actin (α-SMA) (1:200; DAKO, Carpinteria, CA). Fibronectin and α-SMA immunohistochemistry was quantified using Image Pro Plus (Media Cybernetics, Silver Spring, MD) computer image analysis software.

Biochemistry

Mice were injected intraperitoneally 10 to 15 minutes before sacrifice with 0.1 ml of heparin sodium (1000 IU/ml; LEO Pharma Inc., Thornhill, ON, Canada) diluted 1 in 2 with normal saline to prevent blood clotting. Whole blood was collected on ice from the abdominal cavity after the kidneys were harvested. Samples were centrifuged at 3000 rpm at 4°C for 20 minutes, and the plasma was transferred to a new tube and stored at −80°C until analysis. Plasma was used to measure Na^+, K^+, Cl^-, Ca^{2+}, glucose, creatinine, urea, and lactate (Nova Biomedical Stat Profile M7; Centre for Modeling Human Disease, Toronto, ON, Canada, or VITA-TECH, Markham, ON, Canada). Ang I and Ang II concentrations were measured in snap-frozen whole kidney tissue by radioimmunoassay in the Hypertension and Vascular Disease Center Core Laboratory at Wake Forest University School of Medicine, Winston-Salem, NC, as previously described.[12]

Echocardiographic and Hemodynamic Parameters

Mice were anesthetized with isoflurane/oxygen (1/99%), and measurements were made as previously described.[13] Hemodynamic parameters were obtained from the common carotid using a 1.4 French Millar catheter (Millar Inc., Houston, TX) advanced into the proximal aorta and then into the left ventricle. Pressure recordings were filtered at 200 Hz and sampled at 2 kHz (Acq 3.73; BioPac Systems Inc., Goleta, CA). Noninvasive tail-cuff systolic blood pressure (TC-SBP) in the mice was recorded as previously described.[13]

Real-Time Reverse Transcriptase (RT)-PCR

Renal cortical RNA expression levels for the genes reported in this study were quantified by real-time RT-PCR as described previously.[14] In brief, kidney samples from mice were snap-frozen in liquid nitrogen, the cortex was later dissected in an RNA-stabilizing solution (RNAlater; Ambion Inc., Austin, TX), and RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). One μg of total RNA was reverse-transcribed, and RNA expression levels were quantified by real-time RT-PCR using a sequence detection system (Prism 7700; Applied Biosystems Inc., Foster City, CA) as described previously.[15] 18S rRNA was used as the endogenous control. Primers and probes for the reported genes are indicated in Table 1.

Table 2. Whole Animal Data

Parameter	$Ace2^{+/y}Ins2^{WT/WT}$	$Ace2^{-/y}Ins2^{WT/WT}$	$Ace2^{+/y}Ins2^{WT/C96Y}$	$Ace2^{-/y}Ins2^{WT/C96Y}$
BW (g)	25.0 ± 0.4 (n = 16)	24.5 ± 0.6 (n = 19)	23.1 ± 0.4*† (n = 26)	23.4 ± 0.5*† (n = 23)
KW (g)	0.185 ± 0.006 (n = 17)	0.162 ± 0.008 (n = 20)	0.304 ± 0.011*† (n = 26)	0.291 ± 0.017*† (n = 23)
KW/BW	0.0072 ± 0.00015 (n = 26)	0.0063 ± 0.00018 (n = 31)	0.0134 ± 0.00043*† (n = 43)	0.0134 ± 0.00049*† (n = 37)
HW (g)	0.141 ± 0.005 (n = 16)	0.154 ± 0.006 (n = 19)	0.141 ± 0.005 (n = 26)	0.146 ± 0.005 (n = 23)
Plasma glucose (mmol/L)	14.2 ± 1.8 (n = 6)	14.8 ± 0.9 (n = 6)	52.0 ± 5.5*† (n = 6)	57.8 ± 5.7*† (n = 7)
UV (ml)	2.87 ± 0.15 (n = 20)	3.29 ± 0.25 (n = 20)	19.02 ± 0.88*† (n = 33)	39.3 ± 1.5*†‡ (n = 28)
Urinary albumin (μg/day)	31.79 ± 2.52 (n = 20)	37.92 ± 3.71 (n = 20)	178.77 ± 11.91*† (n = 26)	398.78 ± 25.60*†‡ (n = 28)

Body weight (BW), kidney weight (KW), kidney-weight-to-body-weight ratio (KW/BW), heart weight (HW), plasma glucose, 24-hour urine volume (UV), and 24-hour urinary albumin excretion rate were measured upon sacrifice at 3 months of age for all four groups of mice. Data are presented as mean ± SEM with sample sizes indicated in parentheses beside each value.

*$P < 0.05$ compared with $Ace2^{+/y}Ins2^{WT/WT}$.

†$P < 0.05$ compared with $Ace2^{-/y}Ins2^{WT/WT}$.

‡$P < 0.05$ compared with $Ace2^{+/y}Ins2^{WT/C96Y}$.

Western Blot Analysis

Kidney tissue was washed in ice-cold phosphate-buffered saline, sonicated in modified RIPA buffer (150 mmol/L sodium chloride, 50 mmol/L Tris-HCl, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L phenylmethyl sulfonyl fluoride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) for 10 seconds twice, and then incubated on ice for 25 minutes. After centrifugation at 14,000 rpm for 10 minutes, the protein concentration was determined in the supernate. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%), transferred to nitrocellulose membranes, and blocked for 1 hour at room temperature with Tris-buffered saline with 0.1% Tween 20 and 5% nonfat dry milk. After blocking, membranes were incubated with a rabbit polyclonal anti-ACE2 antibody, 1:1000, overnight at room temperature (provided by J.M.P.). Immunodetection was performed with chemiluminescence as previously described after incubation with peroxidase-conjugated anti-rabbit IgG.[16]

Statistical Analysis

Results are expressed as mean ± SEM, unless otherwise specified. Comparisons between multiple groups were performed by one-way analysis of variance. Both parametric and nonparametric tests were used with SPSS software (Chicago, IL), specifically the Fisher's least significant difference and Tamhane's T2 methods. $P < 0.05$ was considered statistically significant.

Results

Whole Animal Studies

Four groups of male mice with a combination of mutant or wild type at the *Acell* and *Ins2* loci were generated:

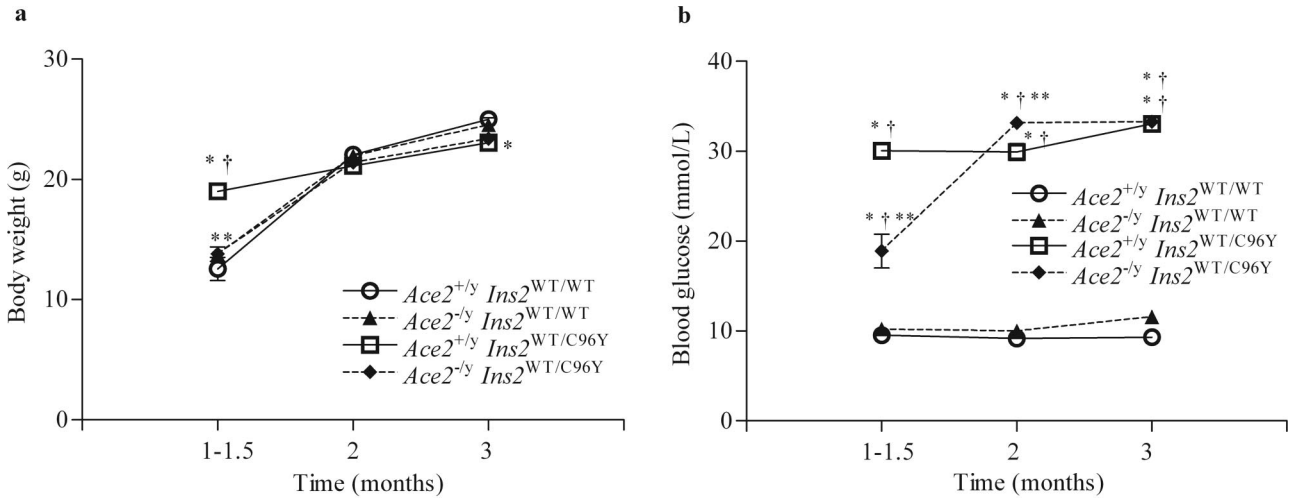

Figure 2. Body weight and blood glucose measurements. **a:** Body weights were measured monthly and the values were similar in the four groups of mice. **b:** Blood glucose was also measured monthly in each group of mice using an Ascensia Breeze glucometer. Both diabetic groups developed early and sustained hyperglycemia compared with the nondiabetic groups. Data are presented as mean ± SEM. *$P < 0.05$ compared with $Ace2^{+/y}Ins2^{WT/WT}$ mice, †$P < 0.05$ compared with $Ace2^{-/y}Ins2^{WT/WT}$ mice, and **$P < 0.05$ compared with $Ace2^{+/y}Ins2^{WT/C96Y}$ mice.

Table 3. Plasma Electrolytes and Renal Function

Electrolyte	*Ace2*$^{+/y}$*Ins2*$^{WT/WT}$	*Ace2*$^{-/y}$*Ins2*$^{WT/WT}$	*Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$	*Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$
Na^+ (mmol/L)	142.8 ± 0.9 (n = 6)	145.2 ± 1.5 (n = 6)	141.3 ± 2.1 (n = 6)	137.4 ± 4.9 (n = 8)
Cl^- (mmol/L)	110.3 ± 0.9 (n = 6)	113.0 ± 1.8 (n = 6)	109.5 ± 1.7 (n = 6)	105.6 ± 2.7† (n = 8)
Ca^{2+} (mmol/L)	0.84 ± 0.08 (n = 4)	1.12 ± 0.02* (n = 3)	0.84 ± 0.04† (n = 4)	0.88 ± 0.06† (n = 4)
Creatinine (μmol/L)	42.7 ± 5.7 (n = 6)	36.7 ± 8.4 (n = 6)	45.3 ± 5.1 (n = 6)	31.9 ± 1.9 (n = 7)
Lactate (mmol/L)	6.12 ± 1.00 (n = 4)	6.07 ± 1.33 (n = 3)	7.33 ± 1.87 (n = 4)	6.00 ± 1.24 (n = 4)
Urea (mmol/L)	10.5 ± 0.6 (n = 6)	11.3 ± 1.1 (n = 6)	15.0 ± 1.6 (n = 6)	16.3 ± 2.1*† (n = 8)

Na^+, Cl^-, Ca^{2+}, creatinine, lactate, and urea concentrations were measured by CMHD or VITA-TECH from plasma collected from the abdominal cavity after sacrifice at 3 months of age. Data are presented as mean ± SEM with sample sizes indicated in parentheses beside each value.
$^*P < 0.05$ compared with *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice.
$^\dagger P < 0.05$ compared with *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ mice.

Ace2$^{+/y}$*Ins2*$^{WT/WT}$ (control C57BL/6J), *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ (ACE2 knockout), *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ (single mutant diabetic Akita), and *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ (double mutant diabetic ACE2 knockout) mice (Figure 1). Whole animal data, including body, heart, and kidney weights, plasma glucose, and 24-hour albumin excretion, are listed in Table 2. Monthly glucometer blood glucose measurements showed that both the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice and *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice developed early and sustained hyperglycemia (Figure 2b), and weight gains were similar (Figure 2a). Likewise, at 3 months, plasma glucose levels in the diabetic groups were significantly greater than in the nondiabetic groups. There was no significant difference between the plasma glucose concentrations in the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ and *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice (Table 2). Despite severe hyperglycemia in the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice (52.0 ± 5.5 mmol/L) and *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice (57.8 ± 5.7 mmol/L), body weights remained similar in all four of the groups (Figure 2a and Table 2).

Kidney weights in both diabetic groups were almost twofold greater than in the *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ or *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ groups (Table 2). As expected, plasma creatinine values were similar in the four groups (Table 3), although there was a trend toward lower values in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice (P = 0.095). The urinary AER increased in the diabetic *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice compared with the nondiabetic *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice, and there was a dramatic additive effect of the deletion of the *Acell* gene on the urinary AER rates in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice (Figure 3). Despite similar blood glucose levels, the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice exhibited a near doubling of the urinary AER compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice. There were no differences in the measures of plasma electrolytes (Table 3).

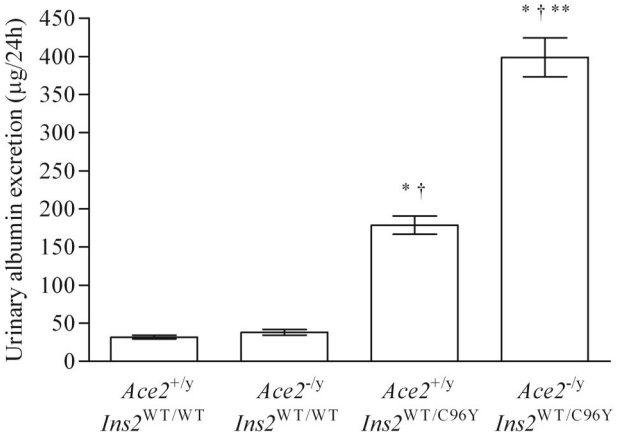

Figure 3. Development of increased urinary AERs in the diabetic mice. The urinary AER was measured with an enzyme-linked immunosorbent assay in 24-hour urine samples collected at 3 months. The AER rate increased in both groups of diabetic mice but the AER was twofold greater in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice at 3 months of age. Data are presented as mean ± SEM. $^*P < 0.05$ compared with *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice, $^\dagger P < 0.05$ compared with *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ mice, and $^{**}P < 0.05$ compared with *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice.

Histomorphometric Studies of the Kidney

Given the marked effect of deletion of the *Acell* gene on the urinary AER, we further sought to determine whether there were differences in kidney structure (Figure 4 and Table 4). Morphometric assessment showed that mean values for the glomerular volume tended to increase in both diabetic groups, but only the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice exhibited a significant increase in glomerular volume compared with the control *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice (Table 4). Mean values for the mesangial matrix score also tended to increase in both diabetic groups. The *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice developed a significant increase in the mesangial matrix score compared with the control *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice (Figure 4). In accord with the light microscopy, the GBM thickness was greater in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice than in the control *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice (Table 4). Moreover, the GBM thickness was also significantly greater in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ diabetic mice than in the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ diabetic mice (Table 4).

To characterize further the mesangial injury, immunohistochemical studies were performed in the four groups of mice (Figures 5 and 6). There was a twofold increase in glomerular fibronectin immunostaining in the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice and a threefold increase in fibronectin immunostaining in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice. Fibronectin immunostaining was also greater in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice (Figure 5). α-Smooth muscle

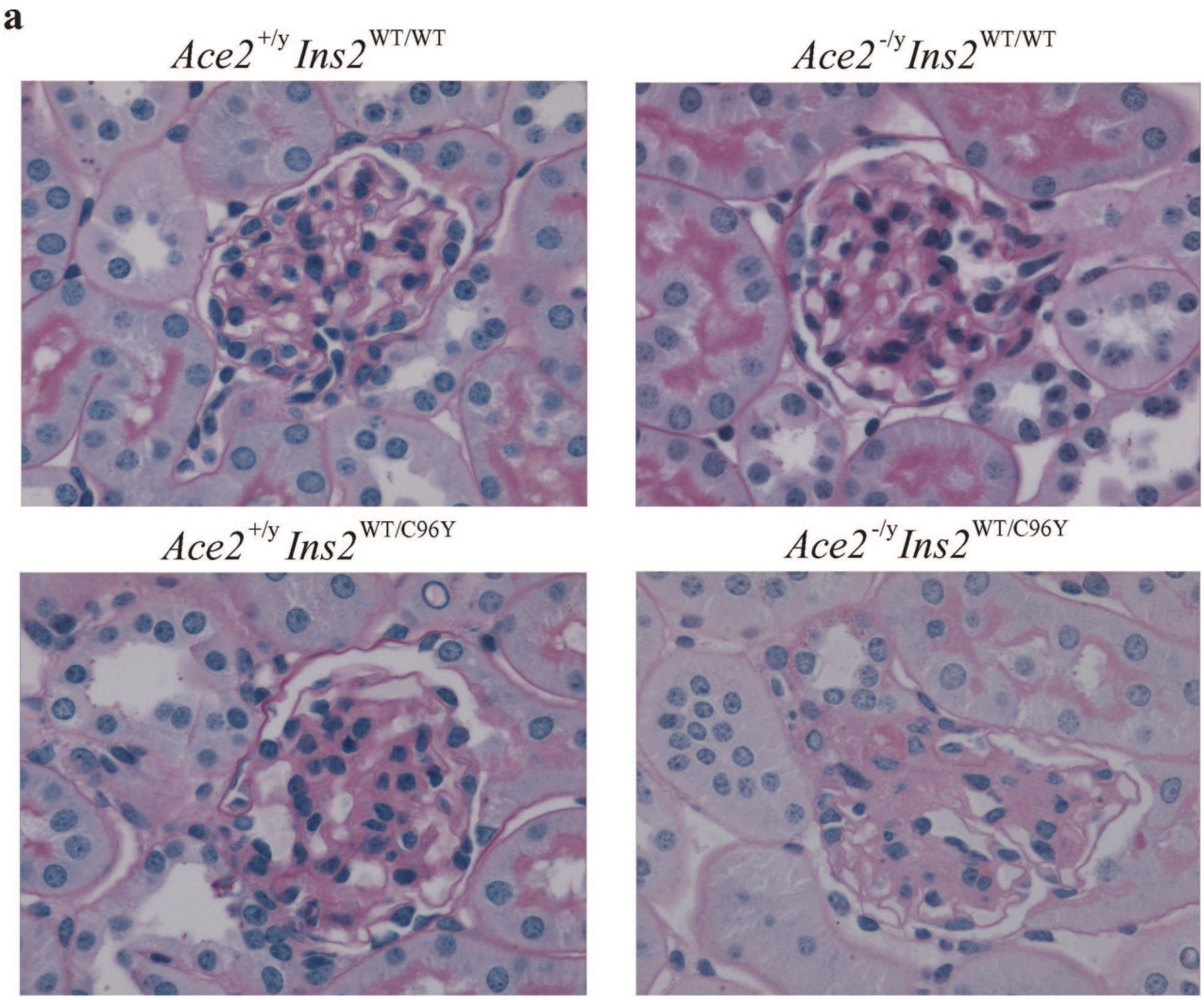

Figure 4. Analysis of the mesangial matrix scores in glomeruli. **a:** Representative light micrographs of PAS-stained kidney sections from each group of mice. **b:** Mesangial matrix scores were generated on a quadrant scale (0 to 4) based on the amount of PAS-positive material in 34 glomerular profiles per animal. The $Ace2^{-/y}Ins2^{WT/C96Y}$ mice were the only diabetic group to develop a significant increase in the mesangial matrix score at 3 months of age compared with the control $Ace2^{+/y}Ins2^{WT/WT}$ mice. Data are presented as mean ± SEM on $n = 6$ per group. $^{*}P < 0.05$ compared with $Ace2^{+/y}Ins2^{WT/WT}$ mice, and $^{\dagger}P < 0.05$ compared with $Ace2^{-/y}Ins2^{WT/WT}$ mice. Original magnifications, ×600.

actin (α-SMA) immunostaining was increased in both of the diabetic groups of mice compared with the $Ace2^{+/y}Ins2^{WT/WT}$ mice, but there was also a marked increase in α-SMA in the $Ace2^{-/y}Ins2^{WT/C96Y}$ mice compared with the $Ace2^{+/y}Ins2^{WT/C96Y}$ mice (Figure 6).

Echocardiographic and Hemodynamic Studies

Blood pressure is an important determinant of diabetic kidney injury. Tail-cuff SBP measures revealed no significant differences in SBP among the various groups of

Table 4. Renal Histomorphometry

Histological parameter	*Ace2*$^{+/y}$*Ins2*$^{WT/WT}$	*Ace2*$^{-/y}$*Ins2*$^{WT/WT}$	*Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$	*Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$
Glomerular volume (μm^3)	116,396 ± 6011 ($n = 6$)	107,523 ± 9270 ($n = 6$)	132,908 ± 6675† ($n = 6$)	153,949 ± 7866*† ($n = 6$)
Mesangial matrix score	1.69 ± 0.15 ($n = 6$)	1.50 ± 0.13 ($n = 7$)	2.06 ± 0.18† ($n = 6$)	2.34 ± 0.15*†‡ ($n = 6$)
GBM thickness (nm)	114.0 ± 0.9 ($n = 2$)	107.9 ± 2.6 ($n = 3$)	115.9 ± 3.0† ($n = 2$)	127.6 ± 1.0*†‡ ($n = 3$)

Glomerular volume, mesangial matrix score (0 to 4), and glomerular basement membrane (GBM) thickness were measured at 3 months. Data are presented as mean ± SEM with sample sizes indicated in parentheses beside each value.

$^{*}P < 0.05$ compared with *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice.

$^{\dagger}P < 0.05$ compared with *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ mice.

$^{\ddagger}P < 0.05$ compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice.

mice nor were there any differences in measures of intra-aortic blood pressure (Table 5). In addition, previous studies have shown that deletion of the *Acell* gene leads to impaired heart function[13] and a marked impairment of cardiac function in the double mutant mice might have accounted for the difference in kidney injury. As shown in Table 5, transthoracic echocardiography revealed no significant differences in heart rate, ventricular dimensions, or myocardial contractility in the four groups of mice. The lack of systolic dysfunction was confirmed by hemodynamic measurements that showed no differences in $(dP/dt)_{max}$ or $(dP/dt)_{min}$ in the four groups of mice (Table 5).

Gene Expression Studies

We analyzed ACE and ACE2 mRNA levels as well as bradykinin B2 receptor mRNA levels in the kidney cortex of the four groups of mice. As expected, ACE2 mRNA levels were not detected in either the *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ mice or the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice. ACE mRNA levels fell in the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice whereas ACE2 mRNA levels increased. There was no further decline in ACE mRNA levels in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice. Bradykinin B2 receptor mRNA levels increased twofold in both diabetic groups compared with the nondiabetic groups (Figure 7). As expected, Western blot analysis of ACE2 protein levels in the whole kidney homogenates confirmed the measures of ACE2 mRNA levels (Figure 8). There was no ACE2 protein expression in the kidneys of *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ and *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice. ACE2 protein expression increased in the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ I mice.

Angiotensin Receptor Blockade

We have reported that Ang II receptor blockade attenuates development of glomerulosclerosis in aging *Ace2*$^{-/y}$ mice.[13] Accordingly, we treated *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice and *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice with the Ang II type 1 receptor blocker (ARB), irbesartan, to determine whether an ARB would reduce the urinary AER. As shown in Figure 9, treatment with the ARB reduced the urinary AER in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice to the same levels observed in the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice. α-SMA immunostaining was also reduced to normal levels in the glomeruli from the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice (α-SMA staining score, 0.01±.005).

The effect of irbesartan on blood pressure was determined in 8-week-old *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ and *Ace2*$^{-/y}$ *Ins2*$^{WT/WT}$ mice. The mice received 50 mg/kg/day as above, and blood pressure measurements were obtained after 4 weeks of treatment. Blood pressure values tended to be greater in the *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice although the differences did not reach statistical significance (Table 6). Treatment with irbesartan reduced aortic SBP, diastolic blood pressure, and mean arterial blood pressure in both groups of mice.

Previous studies have also suggested that deletion of the *Acell* gene leads to increased generation of Ang II in tissue.[13] To relate the functional studies to Ang peptide levels, we measured the Ang peptides, Ang I and Ang II, in whole kidney samples from the four groups of mice (Table 7). The concentrations of Ang I and Ang II were lower in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice compared with the control *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice and *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ mice.

Discussion

The rationale for this study was twofold: first, activation of the RAS plays an important role in the development of experimental and clinical diabetic nephropathy,[1–3] and second, the recent discovery of an ACE homologue, ACE2, has revised our understanding of Ang peptide processing.[4] Tikellis and colleagues[8] were the first to report that ACE2 expression was reduced in the kidneys of rats with longstanding diabetes mellitus, whereas more recently, Ye and colleagues[6] and Wysocki and colleagues[7] reported that there was an early increase in ACE2 expression and activity in the kidneys of the diabetic *db/db* mouse. Taken together, these studies suggested that ACE2 might play an early protective role in the development of diabetic nephropathy and that a decline in expression of ACE2 might contribute to the development of glomerular injury. However, definitive data regarding the role of ACE2 in the pathogenesis of diabetic nephropathy has been lacking.

The development of microalbuminuria is the first functional abnormality that characterizes the natural history of nephropathy in patients with type 1 diabetes mellitus, and the AER is a primary outcome in many clinical studies of diabetic nephropathy.[1,2,17,18] In addition, treatment-induced declines in albuminuria often predict a reduction in the rate of progression of diabetic nephropathy.[18,19] Accordingly, the first goal of the current study was to determine whether deletion of the *Acell* gene would affect

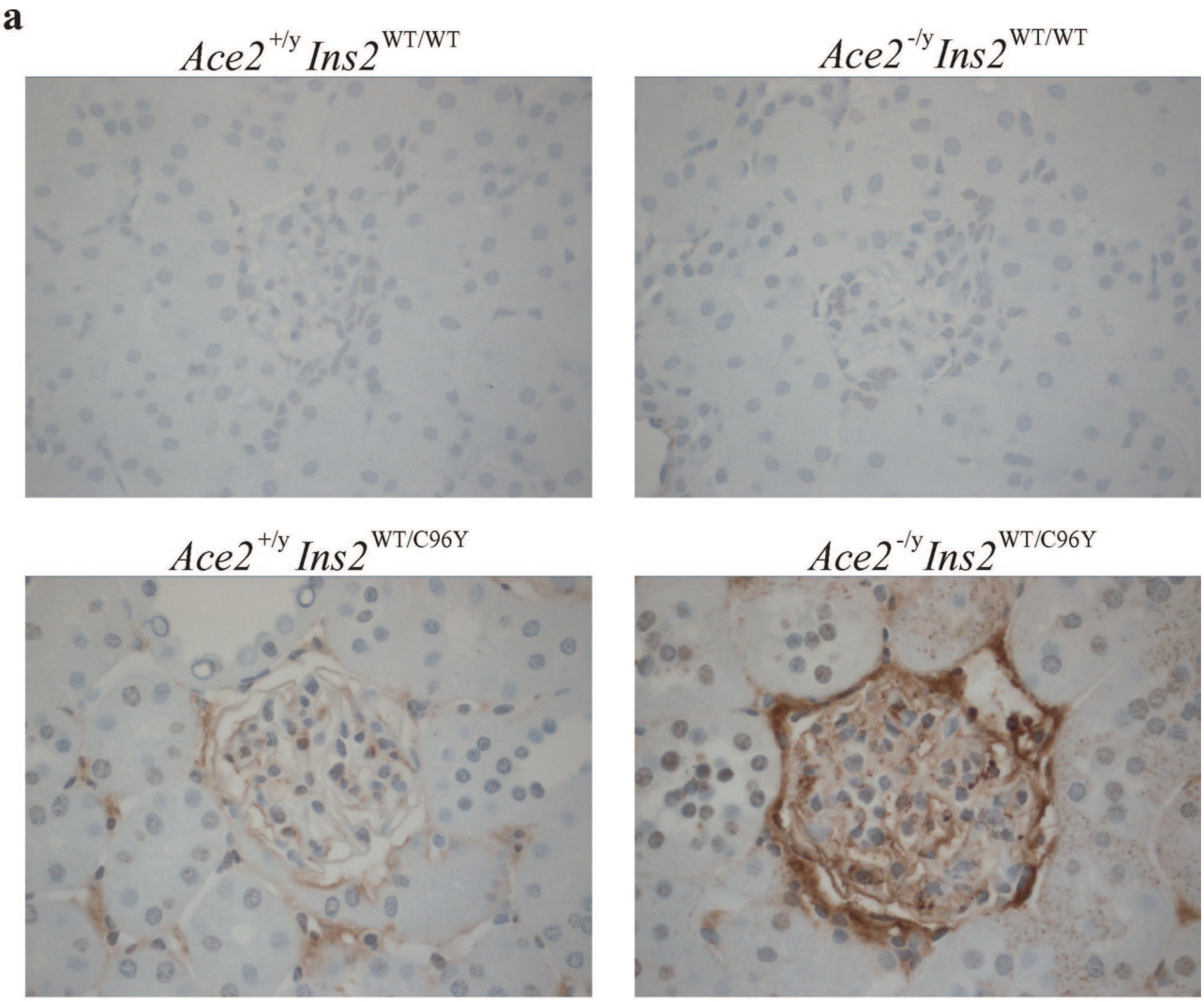

Figure 5. Histological analysis of fibronectin immunostaining in glomeruli. **a:** Representative light micrographs of fibronectin immunostaining in kidney sections from each group of mice. **b:** Fibronectin immunostaining in glomeruli was analyzed by computer image analysis. Fibronectin expression increased in both groups of diabetic mice, but there was a marked increase in fibronectin expression in the glomeruli of the $Ace2^{-/y}Ins2^{WT/C96Y}$ mice compared with the $Ace2^{+/y}Ins2^{WT/C96Y}$ mice at 3 months of age by measuring brown staining pixel density. Data are presented as mean ± SEM. $^{*}P < 0.05$ compared with $Ace2^{+/y}Ins2^{WT/WT}$ mice, $^{\dagger}P < 0.05$ compared with $Ace2^{-/y}Ins2^{WT/WT}$ mice, and $^{**}P < 0.05$ compared with the $Ace2^{+/y}Ins2^{WT/C96Y}$ mice. Original magnifications, ×600.

the urinary AER in the $Ace2^{+/y}Ins2^{WT/C96Y}$ mouse.[20–23] A major finding was that the diabetic $Ace2^{-/y}Ins2^{WT/C96Y}$ mice exhibited a twofold increase in urinary albumin excretion compared with the diabetic $Ace2^{+/y}Ins2^{WT/C96Y}$ mice. In a recent study, Ye and colleagues[24] have reported that pharmacological blockade of ACE2 increases the AER in diabetic *db/db* mice, a model of type 2 diabetes mellitus. Taken together with the cur-

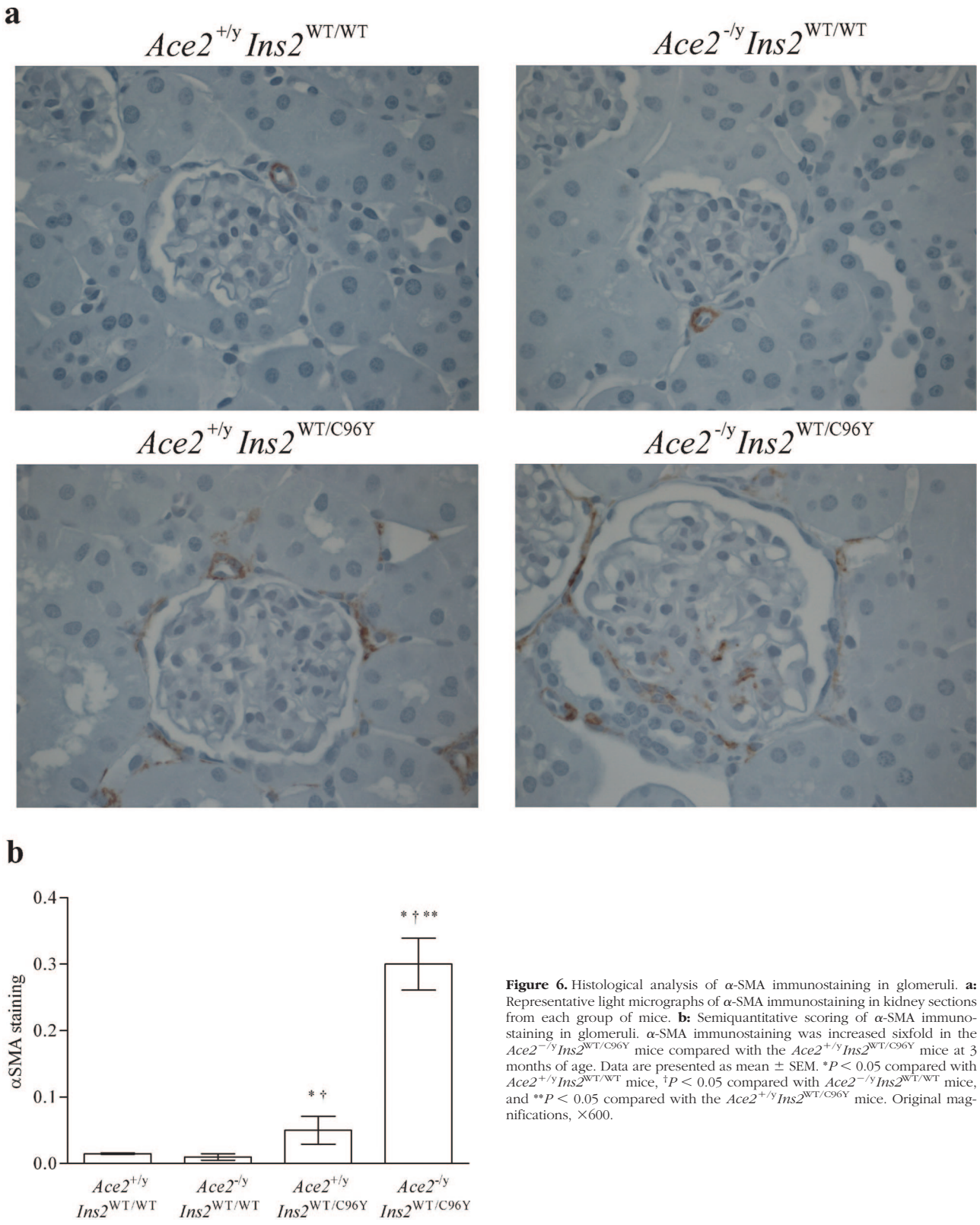

Figure 6. Histological analysis of α-SMA immunostaining in glomeruli. **a:** Representative light micrographs of α-SMA immunostaining in kidney sections from each group of mice. **b:** Semiquantitative scoring of α-SMA immunostaining in glomeruli. α-SMA immunostaining was increased sixfold in the $Ace2^{-/y}Ins2^{WT/C96Y}$ mice compared with the $Ace2^{+/y}Ins2^{WT/C96Y}$ mice at 3 months of age. Data are presented as mean ± SEM. $^{*}P < 0.05$ compared with $Ace2^{+/y}Ins2^{WT/WT}$ mice, $^{\dagger}P < 0.05$ compared with $Ace2^{-/y}Ins2^{WT/WT}$ mice, and $^{**}P < 0.05$ compared with the $Ace2^{+/y}Ins2^{WT/C96Y}$ mice. Original magnifications, ×600.

rent study, this work supports the hypothesis that ACE2 is an important determinant of albuminuria in the diabetic mouse.

Mauer and colleagues[25] have reported structure-function correlations in studies of nephropathy in patients with type 1 diabetes mellitus and therefore a second goal of

Table 5. Echocardiographic and Hemodynamic Parameters

	Ace2$^{+/y}$*Ins2*$^{WT/WT}$	*Ace2*$^{-/y}$*Ins2*$^{WT/WT}$	*Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$	*Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$
HR (bpm)	530 ± 25 (n = 7)	503 ± 19 (n = 6)	498 ± 27 (n = 6)	514 ± 22 (n = 7)
LVEDD (mm)	3.96 ± 0.12 (n = 7)	4.01 ± 0.16 (n = 6)	3.76 ± 0.22 (n = 6)	3.8 ± 0.21 (n = 7)
FS (%)	54.3 ± 3.6 (n = 7)	51.5 ± 4.1 (n = 6)	51 ± 4.3 (n = 6)	50.7 ± 3.9 (n = 7)
VCFc (circ/second)	9.21 ± 0.41 (n = 7)	9.34 ± 0.52 (n = 6)	10.11 ± 0.77 (n = 6)	9.63 ± 0.51 (n = 7)
$(dP/dt)_{max}$ (mmHg/second)	10,621 ± 395 (n = 7)	9886 ± 523 (n = 9)	9537 ± 421 (n = 7)	9282 ± 557 (n = 5)
$(dP/dt)_{min}$ (mmHg/second)	−9448 ± 402 (n = 7)	−8780 ± 482 (n = 9)	−8832 ± 403 (n = 7)	−8539 ± 576 (n = 5)
TC-SBP (mmHg)	98 ± 10 (n = 3)	100 ± 2 (n = 4)	95 ± 2 (n = 2)	93 ± 3 (n = 4)

HR, heart rate; bpm, beats per minute; LVEDD, left ventricular end diastolic dimension; FS, fractional shortening; VCFc, velocity of circumferential fiber shortening; $(dP/dt)_{max/min}$, positive and negative first derivatives of the left ventricular pressure; TC-SBP, tail-cuff systolic blood pressure. Data are presented as mean ± SEM.

this study was to relate the exacerbation of urinary AER in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice to structural changes in the kidneys. Our findings of kidney hypertrophy in the two groups of diabetic mice confirm previous studies.[22,23] The mean kidney weight was numerically greater in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice than in the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice, although the difference did not reach statistical significance. At the light microscopic level, the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice were the only group to exhibit a significant increase in glomerular volume compared with the control *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice at 3 months of age. Glomerular hypertrophy and mesangial matrix expansion are early features of human diabetic nephropathy. Mean values for the glomerular volume and mesangial matrix scores tended to increase in both diabetic groups;the increases were statistically significant in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice. In addition to these light microscopic changes in the glomerular mesangium, an increase in the deposition of extracellular matrix proteins in the glomeruli of the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice was also reflected in the measures of GBM thickness. Exacerbation of AER in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice was also associated with a significantly greater mean value for the GBM thickness, and the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice were the only diabetic mice to exhibit a greater mean value for GBM thickness than the *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice and *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ mice.

Immunohistochemical studies of the kidneys were also performed in the four groups of mice. Both groups of diabetic mice exhibited greater fibronectin immunostaining in glomeruli compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice and the *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ mice, but fibronectin expression was most pronounced in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice. These observations are in accord with previous *in vitro* studies that have related fibronectin expression to glucose-induced activation of protein kinase C in glomerular mesangial cells, and with *in vivo* studies of experimental models of diabetic nephropathy.[26,27] Pharmacological blockade of ACE2 also increases fibronectin expression in the glomeruli of the diabetic *db/db* mouse.[24] We have previously reported[14] that deletion of the *Acell* gene is associated with increased glomerular α-SMA staining in 12-month-old *Ace2*$^{-/y}$ mice, and the rationale for examining α-SMA in the current study is that *de novo* expression of α-SMA reflects activation of the mesangial cell and the development of a myofibroblast-like phenotype that has been linked to Ang II and glomerular injury.[28] Deletion of the *Acell* gene was not associated with any increased glomerular α-SMA immunostaining nor was diabetes mellitus, per se, associated with marked increases in glomerular α-SMA expression in the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice at 3 months of age. However, there was a significant increase in glomerular expression of α-SMA in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice compared with all of the other groups of mice.

A further goal of the current study was to determine the mechanism(s) responsible for the acceleration of the functional and structural changes of diabetic nephropathy in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice. Numerous clinical and experimental studies have shown that glycemic control and blood pressure are critical determinants of the rate of progression of diabetic nephropathy.[1,2,18,29] Although the plasma glucose concentrations at 3 months in the diabetic groups of mice were greater than those reported by Gurley and colleagues[23] (24.8 ± 3.6 mmol/L) and Kakoki and colleagues[22] (38.8 ± 1.9 mmol/L), we observed early and sustained increases in the blood glucose concentrations. At the time of sacrifice, the blood glucose levels were also similar in both groups of diabetic mice, supporting our conclusion that differences in glycemic control were not responsible for the accelerated diabetic kidney injury in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice.

It was also important to evaluate heart function in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice because it has been reported that *Acell*-null mice exhibit decreased cardiac contractility,[13] although recent studies suggest that the phenotype is variable.[30] It was also possible that sustained hyperglycemia might have further exacerbated impairment in cardiac function in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice. Mean values for fractional shortening and velocity of circumferential fiber shortening, measures of heart function, and tail-cuff SBP were similar in the four groups of mice. It is surprising that we did not see a significant cardiac phenotype in the *Acell*-null mice and this may be attributable to the C57BL/6J background. In addition, the failure to detect differences in blood pressure between our groups may have been a function of the number of animals that we studied; blood pressure values did tend to be higher in the *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ mice than in the *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice although the differences did not reach statistical significance. Importantly, tail-cuff SBP was not increased in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice, so we conclude that neither differences in blood pressure nor differences

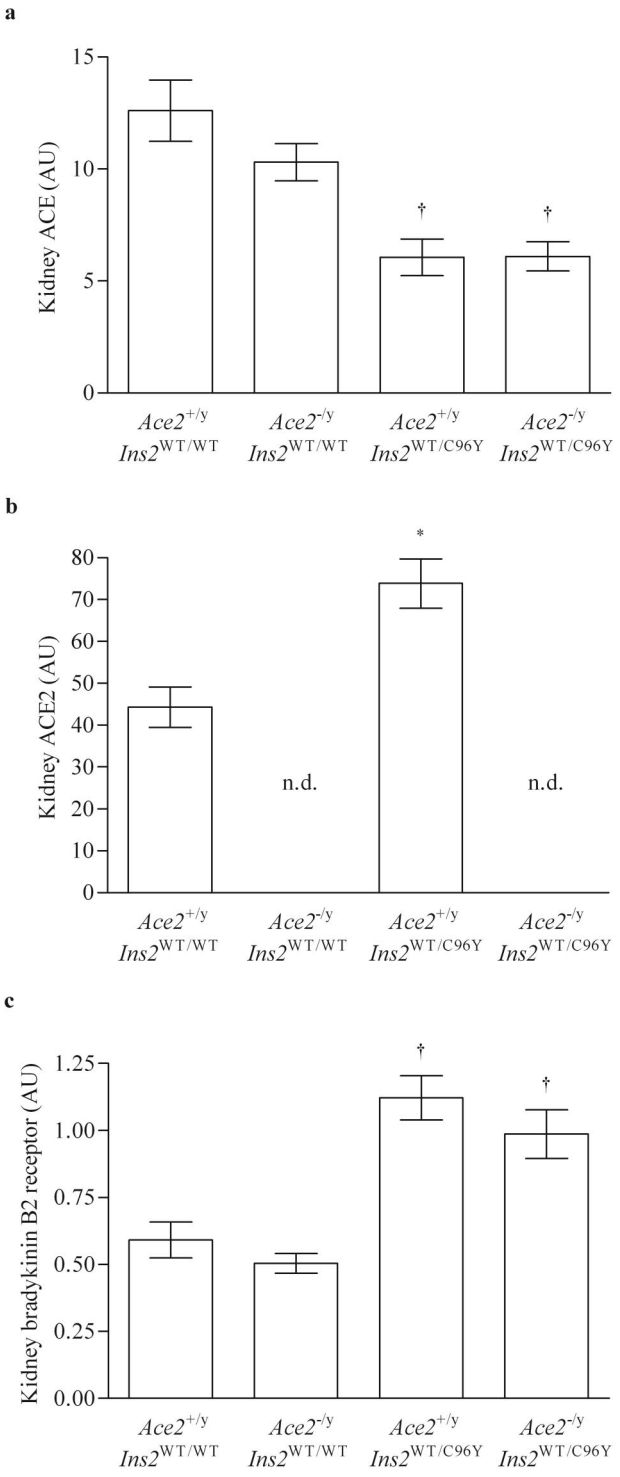

Figure 7. Effect of diabetes mellitus on ACE, ACE2, and the bradykinin B2 receptor expression in the kidney. mRNA levels were measured in the renal cortical tissue by real-time RT-PCR and related to 18S mRNA levels. **a:** Hyperglycemia was associated with a decrease in ACE expression in the kidney cortex of both groups of diabetic mice. **b:** ACE2 expression increased in the $Ace2^{+/y}Ins2^{WT/C96Y}$ mice compared with the $Ace2^{+/y}Ins2^{WT/WT}$ mice and, as expected, was not detected in the mice with deletion of the *AceII* gene. **c:** Bradykinin B2 receptor expression increased in both groups of diabetic mice. Data are presented as mean ± SEM. n.d., not detectable. $^{*}P < 0.05$ compared with $Ace2^{+/y}Ins2^{WT/WT}$ mice, and $^{\dagger}P < 0.05$ compared with both $Ace2^{+/y}Ins2^{WT/WT}$ and $Ace2^{-/y}Ins2^{WT/WT}$ mice. AU, arbitrary unit.

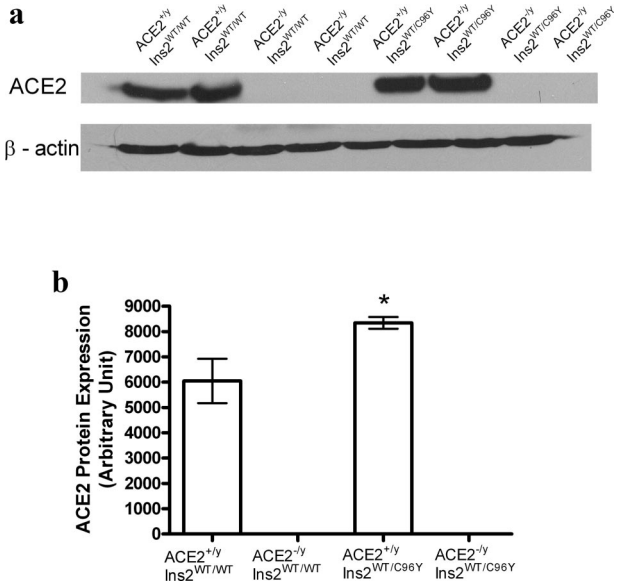

Figure 8. Western blot analysis of ACE2 expression in the kidney. **a:** Western blots for ACE2 and β-actin protein levels in the renal cortical tissue in the four groups of mice. **b:** Densitometry measures of ACE2 protein levels were related to β-actin. Hyperglycemia was associated with an increase in ACE2 expression in the kidney cortex of the $Ace2^{+/y}Ins2^{WT/C96Y}$ mice compared with the $Ace2^{+/y}Ins2^{WT/WT}$ mice. No ACE2 protein expression was detected in the $Ace2^{-/y}Ins2^{WT/WT}$ mice and the $Ace2^{-/y}Ins2^{WT/C96Y}$ mice. Data are presented as mean ± SEM. $^{*}P < 0.05$ compared with $Ace2^{+/y}Ins2^{WT/WT}$ mice.

in heart function were responsible for the accelerated kidney injury in the $Ace2^{-/y}Ins2^{WT/C96Y}$ mice.

Activation of the RAS plays a key role in the progression of clinical and experimental diabetic nephropathy and blockade of the RAS has been shown to have important effects on the rate of progression of diabetic kidney disease.[2,3] ACE2 plays an important role in the process-

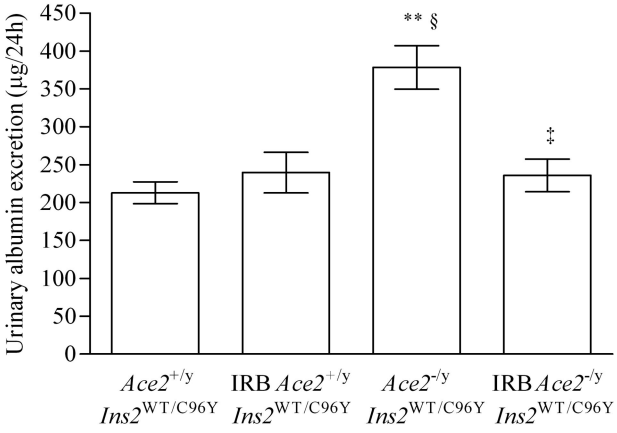

Figure 9. Effect of angiotensin II type 1 receptor blockade on the urinary AER. Four groups of mice were studied: $Ace2^{+/y}Ins2^{WT/C96Y}$ mice and $Ace2^{-/y}Ins2^{WT/C96Y}$, either untreated or treated with the angiotensin II type 1 receptor blocker irbesartan (IRB) from weaning until 3 months of age ($n = 6$ in each group). The urinary AER was measured with an enzyme-linked immunosorbent assay in 24-hour urine samples. The AER was greater in untreated $Ace2^{-/y}Ins2^{WT/C96Y}$ mice compared with untreated $Ace2^{+/y}Ins2^{WT/C96Y}$ mice but this difference was prevented by treatment with IRB. Data are presented as mean ± SEM. $^{**}P < 0.05$ compared with $Ace2^{+/y}Ins2^{WT/C96Y}$ mice, $^{\S}P < 0.05$ compared with IRB $Ace2^{+/y}Ins2^{WT/C96Y}$ mice, and $^{\ddagger}P < 0.05$ compared with $Ace2^{-/y}Ins2^{WT/C96Y}$ mice.

Table 6. Blood Pressure in Mice Treated with Irbesartan (50 mg/kg/day) for 4 Weeks

	Ace2$^{+/y}$*Ins2*$^{WT/WT}$	*Ace2*$^{+/y}$ *Ins2*$^{WT/WT}$ + IRB	*Ace2*$^{-/y}$*Ins2*$^{WT/WT}$	*Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ + IRB
Aortic SBP (mmHg)	114 ± 6 (n = 8)	101 ± 5 (n = 8)*	121 ± 6 (n = 8)	104 ± 5 (n = 8)*
Aortic DBP (mmHg)	78 ± 3 (n = 8)	70 ± 4 (n = 8)	81 ± 4 (n = 8)	69 ± 5 (n = 8)*
MABP (mmHg)	90 ± 4 (n = 8)	80 ± 4 (n = 8)*	94 ± 4 (n = 8)	81 ± 6 (n = 8)*

Eight-week-old *Ace2*$^{+/y}$ *Ins2*$^{WT/WT}$ mice and *Ace2*$^{-/y}$ *Ins2*$^{WT/WT}$ mice were treated with the angiotensin receptor blocker irbesartan (50 mg/kg/day) for 4 weeks before measurement of blood pressure. Data are presented as mean ± SEM.
*$P < 0.05$ compared with untreated mice.

ing of angiotensin peptides in the kidney, as suggested in recent studies by Tikellis and colleagues[8] and Wysocki and colleagues.[7] Indeed, we observed that ACE2 mRNA levels and protein levels increased and ACE mRNA levels decreased in the kidneys of the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice in accord with the findings of Wysocki and colleagues[7] in diabetic *db/db* mice. High glucose concentrations activate the RAS at the level of renin[31,32] and/or angiotensinogen,[31,33,34] and these effects would be expected to increase activity of the RAS in the kidney.[33] Therefore, we predicted that the accelerated kidney injury in *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice might be Ang II-dependent.

To test the hypothesis that exacerbation of diabetic kidney injury in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice was Ang II-dependent, we treated diabetic mice with 50 mg/kg of the Ang II receptor antagonist irbesartan from weaning to 3 months of age. We have previously reported that this dose of irbesartan reduces glomerular injury in aged *Ace2*$^{-/y}$ mice.[14] Another major finding of the current study was that angiotensin receptor blockade with irbesartan reduced the urinary AER in *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice to values similar to those of the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice. This finding is consistent with the hypothesis that acceleration of diabetic kidney injury in *Ace2*$^{-/y}$-*Ins2*$^{WT/C96Y}$ mice is Ang II-dependent. We did not measure blood pressure in the irbesartan-treated diabetic mice although we found that 50 mg/kg/day of irbesartan was sufficient to lower blood pressure in both *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice and *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ mice.

To relate our findings further to activation of the RAS, we measured kidney angiotensin peptide levels in the four groups of mice. We predicted that the Ang II levels would be greatly increased in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice.[30,35] Surprisingly, we found that Ang I levels and Ang II levels were not increased in the kidneys of *Ace2*$^{+/y}$-*Ins2*$^{WT/C96Y}$ mice compared with *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ mice and that deletion of the *ace2* gene did not lead to an increase in Ang II concentrations in samples of whole kidney from the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice. Moreover, we could not confirm that Ang I and Ang II levels were increased in the *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ kidneys compared with the control *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$ kidneys, as previously reported.[13] This may be attributable to the C57BL/6J background, as recently suggested by Gurley and colleagues.[30]

It is possible that whole kidney Ang peptide levels did not accurately reflect the local microenvironment of glomeruli or efferent arterioles in our mice. For example, kinetic models of Ang II production and distribution in the renal cortex predict that AT_1 receptors in the glomerulus respond primarily to arterially delivered Ang II, whereas peritubular AT_1 receptors bind mostly to locally generated Ang II, in a paracrine manner.[36,37] Moreover, the normalization of glomerular α-SMA expression by irebsartan that we observed further supports the hypothesis that there was increased Ang II activity in the glomeruli of the in *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice.

Two additional mechanisms of glomerular injury have been reported recently in studies of the *Ins2*$^{WT/C96Y}$ mouse. Kakoki and colleagues[22] found that deletion of the bradykinin B2 receptor (BKB2R) also accelerated glomerular injury in the *Ins2*$^{WT/C96Y}$ mouse. We therefore measured BKB2R mRNA levels in the kidneys of our *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice and *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice to determine whether deletion of the *Acell* gene had an unexpected effect on BKB2R expression in the kidney. We observed a near doubling in BKB2R mRNA levels in the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice, in accord with Kakoki and colleagues,[22] whereas deletion of the *Acell* gene did not affect BKB2R mRNA levels. A recent study by Susztak and colleagues[38] reported that podocyte apoptosis occurred with the onset of diabetes in *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice and preceded the development of albuminuria, whereas Liebau and colleagues[39] found that Ang II increases apoptosis in human podocytes. Taken together, an increase in Ang II coupled with hyperglycemia in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice might induce greater levels of podocyte apoptosis, accounting for the accelerated nephropathy seen in this group. In this regard, ACE2 is expressed on glomerular podocytes as well as mesangial

Table 7. Angiotensin Peptide Measures in Whole Kidney

	Ace2$^{+/y}$*Ins2*$^{WT/WT}$	*Ace2*$^{-/y}$*Ins2*$^{WT/WT}$	*Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$	*Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$
Ang I (fmol/mg protein)	7.84 ± 0.77 (n = 5)	7.61 ± 0.70 (n = 6)	6.43 ± 0.63 (n = 6)	5.04 ± 0.91*† (n = 6)
Ang II (fmol/mg protein)	30.5 ± 3.5 (n = 5)	30.8 ± 4.6 (n = 6)	22.7 ± 3.6 (n = 6)	18.5 ± 3.3*† (n = 6)

Whole kidney Ang peptide levels at 3 months. Ang I and Ang II concentrations were measured in kidney tissue in the four groups of mice at 3 months of age (n = 5 in each group). Data are presented as mean ± SEM.
*$P < 0.05$ compared with *Ace2*$^{+/y}$*Ins2*$^{WT/WT}$.
†$P < 0.05$ compared with *Ace2*$^{-/y}$*Ins2*$^{WT/WT}$ mice.

cells.[24] Further studies will be necessary to determine whether deletion of the *Acell* gene leads to more podocyte apoptosis in *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice.

Our understanding of the role of ACE2 in human kidney disease is incomplete. In a recent study from Japan, Konoshita and colleagues[40] compared ACE2 mRNA levels in the kidneys of a group of diabetic patients with a mixed group of patients with primary glomerular disease, nephrosclerosis, and lupus nephritis. They reported that ACE2 mRNA levels were similar in kidneys from these two disease groups, but they did not compare the levels of expression to a relatively normal group of kidneys.[40] More recently, Lely and colleagues[41] reported ACE2 immunostaining was uniformly increased in the glomeruli and peritubular capillaries of the kidney in a variety of renal diseases that included diabetic nephropathy. Because deletion of the *Acell* gene was associated with accelerated kidney injury in our mice, it is tempting to speculate that ACE2 is renoprotective and that reduced ACE2 expression might contribute to the progression of kidney disease.

In summary, we conclude that ACE2 is an important determinant of diabetic kidney injury. This conclusion is based on structure-function studies in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice that show an increased urinary AER, in association with increased glomerular volume, increased mesangial matrix expansion, increased fibronectin and α-SMA expression, and increased GBM thickening compared with the *Ace2*$^{+/y}$*Ins2*$^{WT/C96Y}$ mice. Differences in blood glucose levels, blood pressure, and heart function do not account for the accelerated kidney injury. The reduction in urinary AER by Ang II type 1 receptor blockade in the *Ace2*$^{-/y}$*Ins2*$^{WT/C96Y}$ mice suggests that Ang II is responsible for exacerbation of kidney injury in this experimental model.

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