Human Recombinant ACE2 Reduces the Progression of Diabetic Nephropathy

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OBJECTIVE—Diabetic nephropathy is one of the most common causes of end-stage renal failure. Inhibition of ACE2 function accelerates diabetic kidney injury, whereas renal ACE2 is downregulated in diabetic nephropathy. We examined the ability of human recombinant ACE2 (hrACE2) to slow the progression of diabetic kidney injury.

RESEARCH DESIGN AND METHODS—Male 12-week-old diabetic Akita mice (Ins2 WT/C96Y) and control C57BL/6J mice (Ins2 WT/WT) were injected daily with placebo or with hrACE2 (2 mg/kg, i.p.) for 4 weeks. Albumin excretion, gene expression, histomorphometry, NADPH oxidase activity, and peptide levels were examined. The effect of hrACE2 on high glucose and angiotensin II (ANG II)–induced changes was also examined in cultured mesangial cells.

RESULTS—Treatment with hrACE2 increased plasma ACE2 activity, normalized blood pressure, and reduced the urinary albumin excretion in Akita Ins2 WT/C96Y mice in association with a decreased glomerular mesangial matrix expansion and normalization of p47phox and smooth muscle actin and collagen III expression. Human recombinant ACE2 increased ANG 1–7 levels, lowered ANG II levels, and reduced NADPH oxidase activity. mRNA levels for p47phox and NOX2 and protein levels for protein kinase Co (PKCo) and PKCβ1 were also normalized by treatment with hrACE2. In vitro, hrACE2 attenuated both high glucose and ANG II–induced oxidative stress and NADPH oxidase activity.

CONCLUSIONS—Treatment with hrACE2 attenuates diabetic kidney injury in the Akita mouse in association with a reduction in blood pressure and a decrease in NADPH oxidase activity. In vitro studies show that the protective effect of hrACE2 is due to reduction in ANG II and an increase in ANG 1–7 signaling.

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C hronic kidney disease is recognized as an increasing global public health problem due in part to the increasing prevalence of diabetes (1–3). Activation of the renin-angiotensin system (RAS) and the generation of angiotensin II (ANG II) play an important pathogenic role in diabetic nephropathy, and blockade of the RAS attenuates the development of diabetic kidney injury (4–8). The discovery of a homologue of the classical ACE, ACE2, has introduced a new enzyme in ANG peptide metabolism (9–12). Like ACE, ACE2 is membrane bound, but it is a monocarboxypeptidase that generates ANG (1–7) from the octapeptide ANG II (9,10,12,13). As such, ACE2 serves as an endogenous negative regulator of the renin-angiotensin system.

In animal models of diabetes, early increases in ACE2 mRNA levels, protein expression, and ACE2 activity occur (14,15), whereas ACE2 mRNA and protein levels have been found to decrease in older streptozotocin-induced diabetic rats (16). Loss of ACE2 is associated with age-dependent glomerulosclerosis and albuminuria (17) and exacerbation of diabetic kidney injury in Akita mice (18) and is preventable by angiotensin type 1 (AT1) receptor blockade. In patients with type 2 diabetes, glomerular and tubular ACE2 expressions are reduced in the setting of increased ACE expression (19,20). Taken together, these studies suggest that ACE2 may play an early protective role against the development of diabetic nephropathy (18,21,22).

We hypothesized that treatment with human recombinant ACE2 (hrACE2) will target the diabetic glomerulus and slow progression of diabetic nephropathy in the Akita mouse (Ins2 WT/C96Y), a model of type 1 diabetes.

RESEARCH DESIGN AND METHODS

Experimental animals and protocol. C57BL/6J and diabetic heterozygous Akita (Ins2 WT/C96Y) mice were purchased from The Jackson Laboratory and bred in our animal facility. Throughout the period of study, animals were provided with free access to water and standard 18% protein rodent chow (Harlan Teklad, Madison, WI). Ins2 WT/C96Y (Akita) and Ins2 WT/WT mice were treated from 3 months of age with daily injections of placebo or human recombinant ACE2 at a daily dose of 2 mg/kg for 4 weeks. Twenty-four–hour urine volumes were collected at the end of 4 months and animals were killed. All experiments were conducted in accordance with the Canadian Council of Animal Care and Institutional Guidelines.

Generation and characterization of human recombinant ACE2. The extracellular domain of human ACE2 (amino acid residues 1–740, molecular wt = 101 kDa) (9) was expressed recombinantly in CHO cells under serum-free conditions in a chemically defined medium. The expression product was purified to homogeneity by applying a capture step on a DEAE Sepharose anion exchanger resin (Pharmacia Biotech AB, Uppsala, Sweden). The eluted fractions containing the expression product were submitted to a polishing step on a Superdex 200 gel filtration column (Pharmacia Biotech AB). The expression product was compared with the commercially available ACE2 standard 933-ZN (R&D Systems, Minneapolis, MN). Chemical and...
imunological properties of both products were almost identical, although hrACE2 showed a 93% enzymatic activity with Mca-APK-(Dnp)-OH substrate in comparison with hrACE2 standard 93% ZN (R&D Systems). The enzymatic turnover of hrACE2 with ANG II substrate was 5.2 ± 0.1 μmol · mg⁻¹ · min⁻¹, and the elimination half-life of hrACE2 was 10.4 h in rhesus monkeys. The purity of the expressed protein was 90.90% measured by high-performance liquid chromatography.

**Plasma ACE2 activity and detection of anti-ACE2 antibodies.** Plasma collected from mice injected with hrACE2 (2 mg/kg, i.p.) for 2 weeks were stored at −80°C. The enzymatic activity of hrACE2 in plasma samples was measured by its ability to cleave the fluorescent peptide substrate Mca-Ala-Ala-Pro-Lys-(4-acetylaminofluorescein)-AMC (AMC). The fluorescence was measured in 3.5 diluted samples (final assay dilution) using excitation and emission wavelengths of 320 and 430 nm, respectively, in presence of 100 μMol/l substrate in 50 mMol/l MES, 300 mMol/l NaCl, 10 μMol/l ZnCl₂, and 0.01% Brij-30 at pH 6.5. Evaluation was performed by comparing the maximal slope of the fluorescence/time curve to respective maximal slopes of a serial hrACE2 dilution in normal mouse plasma. The response to the specific peptide ACE2 inhibitor DX600 (23) (Phoenix Pharmaceuticals, Burlingame, CA) on the ACE2 activity in murine plasma was also examined.

Serum samples of mice were analyzed using an ACE2 antigen-specific enzyme-linked immunosorbent assay (ELISA) recognizing total anti-ACE2-specific IgG. Recombinant human soluble ACE2 was presented as antigen, coated at 10 μg/ml onto Maxisorp adsorption plates (Nunc, Vedbaek, Denmark) diluted in coating buffer. Remaining active groups were blocked by incubating plates in 2% skim milk (Difco) in PBS. Induced antibodies were detected by their constant domains using a rabbit anti-mouse IgG or a rabbit anti-mouse IgM peroxidase-labeled antibody (Zymed). Staining was performed by o-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich) in staining buffer (PAA Laboratories) using H₂O₂ as substrate according to the manufacturer’s instructions. Absorbance at 492 nm was measured using 620 nm as reference wavelength. Quantification was performed by comparison with a commercially available monoclonal mouse anti-human ACE2 antibody (R&D Systems).

**Blood glucose, urinary albumin excretion, and tail-cuff blood pressure measurements.** Blood glucose levels were obtained weekly between 8:30 and 10:30 AM using an Ascensia Breeze glucometer (Bayer, Toronto, ON, Canada), and hyperglycemia was stable and sustained in Ins2²/Yc/rC567S mice, as previously reported (18). Twenty-four-hour urine collections were obtained from mice prior to sacrifice by housing them in individual mouse metabolic cages (Nalgene, model 650-0311; Nalge Nunc International, Rochester, NY) with free access to water and rodent mash. Urinary albumin concentration was measured using an indirect competitive ELISA according to the manufacturer’s instructions (Albuwell M; Exocell, Philadelphia, PA).

For the measurement of tail-cuff systolic blood pressure (TC-SBP), conscious mice were placed in the restrainers and their body temperature was maintained at ~34°C by the warming chamber. The ITC tail-cuff sensor containing both the inflating cuff and the photoelectric sensor was placed on the tail and attached to the restrainer. The cuff was inflated to a pressure of 200 mmHg and then deflated slowly. Upon reappearance of pulse signals, TC-SBP data from the IITC amplifier were recorded, analyzed, and reported by the ITC BioScience Blood Pressure System, Woodland Hills, CA. The mice were trained on three occasions before actual recordings were made, and the corresponding TC-SBPs were averaged from three readings and used for the averaged comparisons.

**Histopathology and electron microscopy.** Kidneys were harvested for pathological examination and one section was fixed in 10% neutral-buffered formalin (Nalgene) on the ACE2 activity in murine plasma was also examined.

Serum samples of mice were analyzed using an ACE2 antigen-specific enzyme-linked immunosorbent assay (ELISA) recognizing total anti-ACE2-specific IgG. Recombinant human soluble ACE2 was presented as antigen, coated at 10 μg/ml onto Maxisorp adsorption plates (Nunc, Vedbaek, Denmark) diluted in coating buffer. Remaining active groups were blocked by incubating plates in 2% skim milk (Difco) in PBS. Induced antibodies were detected by their constant domains using a rabbit anti-mouse IgG or a rabbit anti-mouse IgM peroxidase-labeled antibody (Zymed). Staining was performed by o-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich) in staining buffer (PAA Laboratories) using H₂O₂ as substrate according to the manufacturer’s instructions. Absorbance at 492 nm was measured using 620 nm as reference wavelength. Quantification was performed by comparison with a commercially available monoclonal mouse anti-human ACE2 antibody (R&D Systems).

**RESULTS**

**Human recombinant ACE2 increases serum ACE2 activity and reduces urinary albumin excretion.** Male Ins2²/Yc/rC567S (control C57BL/6J mice) and Ins2²/Yc/rC567S (mutant diabetic Akita mice) were studied at 3 months of age (18,26). Whereas plasma ACE2 activity in Akita mice injected with placebo was undetectable, daily injection of 2 mg/kg of hrACE2 for 2 weeks resulted in measurable serum ACE2 activity of 3,138 ± 721 fluorescence unit/min (n = 6) in Akita mice that was equivalent to 7.14 ± 2.1 μg/ml of hrACE2 (n = 6). The specific ACE2 inhibitor, DX600 (1 μmol/l), suppressed 95 ± 4% of the murine plasma ACE2 activity (n = 3). We hypothesized that the large size of hrACE2 and the increased serum ACE2 activity would target the diabetic glomeruli. Treatment with hrACE2 for 4 weeks reduced the urinary albumin excretion rate by 60% in the diabetic Akita mice (Ins2²/Yc/rC567S) compared with the placebo-treated diabetic Akita mice (Fig. 1A and B). There were no significant differences in the plasma glucose concentrations of the Ins2²/Yc/rC567S + placebo and Ins2²/Yc/rC567S + hrACE2 mice (Fig. 1C). Despite severe hyperglycemia in the Ins2²/Yc/rC567S mice, body weights were similar in all four groups of mice (Table 1).

Assessment of TC-SBP in conscious mice revealed mild hypertension in the Akita mice (Fig. 1D) that declined over a 4-week period in response to daily administration of hrACE2 (Fig. 1E). Human recombinant ACE2 did not affect the serum creatinine concentrations or potassium levels (Table 1).

**Recombinant human ACE2 reduces mesangial matrix expansion.** Given the marked protective effect of hrACE2 on the urinary albumin excretion in the diabetic mice, we sought to relate this functional change to kidney histomorphology. As expected, kidney hypertrophy (Table 1) was associated with an increase in glomerular volume in the Ins2²/Yc/rC567S + placebo mice compared with the control Ins2²/Yc/rC567S mice, and glomerular volume was reduced by hrACE2 treatment (Fig. 2A and C). In accordance with
the light microscopic changes, increased glomerular basement membrane (GBM) thickness in the Akita Ins2WT/WT mice was also significantly reduced in response to hrACE2 treatment (Fig. 2B and D). Diabetic nephropathy is characterized by an accumulation of extracellular matrix proteins in the glomerular mesangium. A semiquantitative and blinded assessment of the mesangial matrix expansion showed a significant increase in the diabetic Akita mice that was reduced by treatment with hrACE2 (Fig. 2E).

TABLE 1
Morphometry and plasma biochemistry in 4-month-old mice

|                  | Ins2WT/WT + placebo | Ins2WT/C96Y + hrACE2 | Ins2WT/WT + placebo | Ins2WT/C96Y + hrACE2 |
|------------------|----------------------|-----------------------|----------------------|-----------------------|
|                  | n                    |                       | n                    |                       |
| BW (g)           | 25.0 ± 1.2           | 25.3 ± 1.4            | 23.1 ± 0.8           | 23.4 ± 1.3           |
| KW (g)           | 0.145 ± 0.06         | 0.151 ± 0.08          | 0.262 ± 0.09*        | 0.254 ± 0.07*        |
| KW/BW (mg/g)     | 0.72 ± 0.15          | 0.63 ± 0.18           | 1.12 ± 0.23*         | 1.10 ± 0.29*         |
| KW/TL (mg/mm)    | 7.25 ± 0.83          | 7.14 ± 0.91           | 11.23 ± 1.65*        | 10.64 ± 1.47*        |
| Plasma K⁺ (mM)   | 4.12 ± 0.32          | 4.5 ± 0.36            | 4.58 ± 0.41          | 4.2 ± 0.46           |
| Creatinine (µM)  | 42.7 ± 5.7           | 36.7 ± 8.4            | 45.3 ± 5.1           | 33.7 ± 8.9           |

Data are means ± SEM. *P < 0.05 compared with corresponding nondiabetic control group. BW, body weight; KW, kidney weight; TL, tibial length.
Macrophage and neutrophil infiltration in the kidneys (Fig. 3E and F). There was no evidence of glomerular or tubulointerstitial infiltration by macrophages or neutrophils in the untreated and treated diabetic Akita mice. In addition, the expression profiles of the proinflammatory cytokines, tumor necrosis factor-α, interleukin-1β, and interleukin-6, and the chemokine, monocyte chemoattractant protein-1, were similar in all four groups of mice (supplementary Table 2).

Recombinant human ACE2 reduces ANG II levels in the plasma and renal cortex. Plasma ACE2 activity was increased in the treated mice, so we measured plasma and renal cortical levels of ANG II, a substrate for ACE2 and ANG 1–7, a product of ACE2, in response to the exogenous hrACE2. In the Akita mice, plasma and renal cortical ANG II levels were significantly reduced by 4 weeks of hrACE2 treatment (Fig. 4A). Consistent with the biochemical action of ACE2, renal ANG 1–7 levels were increased after 4 weeks of treatment with hrACE2. There was a numeric increase in plasma ANG 1–7 levels in the treated mice but the difference did not reach statistical significance (P = 0.092) (Fig. 4B). Renal cortical expression of ace (Fig. 4C) was reduced in the diabetic mice, whereas expression of ace2 (Fig. 4D) was increased in the diabetic Akita mice. The administration of hrACE2 did not influence ace and ace2 expression levels in the kidney, suggesting that the treatment-induced changes in peptide levels were due to exogenous ACE2 activity rather than changes in endogenous kidney expression of the key angiotensin processing enzymes. Similarly, the expression of other components of the RAS known to play a key role in diabetic nephropathy such as the AT1 receptor (Fig. 4E), AT2 receptor (Fig. 4F), bradykinin2 receptor (Fig. 4G), and the Mas receptor (supplementary Table 2) was not influenced by hrACE2 treatment. Consistent with the measures of mesangial matrix expansion, the mRNA expression of the ANG II–sensitive genes fibronectin (Fig. 4H) and pro–collagen III α-1 (Fig. 4I) was increased in Akita diabetic mice and normalized by treatment with hrACE2.

Increased NADPH oxidase activity and PKC expression were suppressed by hrACE2. Increased renal NADPH oxidase activity and activation of the PKC system...
play key roles in the pathophysiology of diabetic nephropathy (5,28–30). Given the protective effect of hrACE2 on diabetic kidney injury, we examined the effect of hrACE2 treatment on renal cortical NADPH oxidase activity and the protein expression of PKCα and PKCβ1 isoforms. In the diabetic Akita mice, renal cortical NADPH activity based on the lucigenin chemiluminescence assay was significantly increased compared with nondiabetic mice (Fig. 5A) in association with increased renal cortical mRNA expression of the NADPH oxidase subunits, NOX2 (gp91phox) (Fig. 5B) and p47phox (Fig. 5C). The cortical expression of the other NADPH subunits including NOX1, NOX4, p22phox, p40phox, and p67phox was not significantly altered in our diabetic model (supplementary Table 3).
Treatment with hrACE2 normalized NADPH oxidase activity and mRNA expression of NOX2 and p47\(^{phox}\) subunits in the diabetic mice (Fig. 5A–C). In the diabetic Akita mice, protein levels of PKC\(\alpha\) (Fig. 5D and E) and PKC\(\beta\)1 (Fig. 5D and F) increased threefold compared with nondoabetic C57BL/6J mice and this effect was attenuated by hrACE2 treatment.

**Human recombinant ACE2 reduces high glucose and ANG II–induced NADPH oxidase activity in mesangial cells:** evidence for the potential role of ANG 1–7.

To address mechanisms responsible for the protective effect of hrACE2 in the diabetic mice, we used cultured primary rat mesangial cells to study the effects of hrACE2 on high glucose and ANG II–induced dihydroethidium fluorescence (DHF) fluorescence and NADPH oxidase activity. High glucose–induced DHF fluorescence was attenuated by both hrACE2 and ANG 1–7 (Fig. 6A–D). NADPH oxidase was also activated by high glucose (Fig. 6E), and this effect was attenuated by pretreatment with rhACE2 (Fig. 6F). As an osmotic control, D-mannitol did not activate NADPH oxidase (Fig. 6E). Consistent with the ability of hrACE2 to metabolize ANG II, hrACE2 also suppressed ANG II–induced DHF fluorescence (Fig. 6E) and reduced NADPH oxidase activity in a dose-dependent manner (Fig. 6F) in mesangial cells. Pretreatment with the specific ACE2 inhibitor, DX600 (1 \(\mu\)mol/L), prevented the ability of hrACE2 to suppress ANG II–mediated activation of NADPH oxidase (Fig. 6F). High glucose concentrations can activate the intrarenal RAS and increase the generation of ANG II in mesangial cells (31–33), so we studied the

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**FIG. 4.** Human recombinant ACE2 alters angiotensin peptide metabolism without a differential impact on expression of the genes of the renin-angiotensin system while normalizing matrix gene expression in diabetic Akita mice. A and B: Reduction in plasma and renal cortical ANG II levels (A) and increases in plasma and renal cortical ANG 1–7 levels (B) in diabetic Akita mice after treatment with hrACE2. n = 10 for placebo group and n = 12 for hrACE2–treated group. *P < 0.05 compared with corresponding placebo group using Student \(t\) test. C–G: Decreased renal cortical ace (C) and increased ace2 (D) expression, unaltered ANG II type 1 receptor, AT1R (E), and type 2 receptor, AT2R (F), expression, and increased bradykinin type 2 receptor, B2R (G), expression in Akita mice were not affected by treatment with hrACE2. n = 8 for placebo groups; n = 10 for hrACE2 groups. *P < 0.05 compared with corresponding Ins2WT/WT group using Student \(t\) test. H and I: Increased renal cortical expression of extracellular matrix genes, fibronectin (H) and pro–collagen III \(\alpha\)-1 (I), in diabetic Akita mice was suppressed in response to hrACE2. n = 8 for placebo groups; n = 10 for hrACE2 groups. #P < 0.05 compared with placebo + Ins2WT/C96Y group using ANOVA and multiple comparison testing.
effects of hrACE2 on high glucose–induced NADPH oxidase activity in mesangial cells pretreated with either the ANG II type I receptor antagonist, losartan, or the Mas receptor peptide antagonist, D-Ala7-ANG 1–7 (34). Treatment with the ANG II type 1 receptor antagonist attenuated the high glucose–induced increase in NADPH oxidase activity with hrACE2, leading to incremental suppression (Fig. 6 K).

The attenuation of high glucose–induced NADPH oxidase activity by hrACE2 was partially prevented by the Mas receptor antagonist, D-Ala7-ANG 1–7, suggesting that part of the effect was mediated by ANG 1–7 (Fig. 6 L). Taken together, these results support the hypothesis that the protective effect of hrACE2 is mediated, at least in part, by a reduction in ANG II and an increase in ANG 1–7 and that together these changes reduce oxidative stress in the diabetic kidney.

### DISCUSSION

Diabetic nephropathy continues to be the most common cause of end-stage renal disease in North America. Activation of the RAS and ANG II play an important role in the development of experimental and clinical diabetic nephropathy, and blockade of the RAS in both experimental and clinical diabetes attenuates the development of diabetic kidney injury (6–8,18). However, ACE inhibitors and angiotensin receptor blockers provide only partial long-term benefits in patients with type 1 (35) and type 2 (6,7,36) diabetes. The recent discovery of an ACE homologue, ACE2, has revised our understanding of the renin-angiotensin system (11,12,37). In a long-standing diabetic rat model, renal ACE2 expression is reduced (16), whereas there is an early increase in ACE2 expression and activity...
in the kidneys of the diabetic db/db (15) and Akita (18) mice. Deletion of the AceII gene and pharmacological inhibition of ACE2 is associated with accelerated glomerular injury in Akita diabetic mice (18) and in streptozocin-induced diabetes (38,39), providing definitive evidence that ACE2 is renoprotective and that reduced ACE2 activity contributes to the progression of kidney disease (19,20). Kidney disease in patients with type 2 diabetes is associated with a reduction in ACE2 mRNA and protein expression (20). Accordingly, we evaluated the ability of hrACE2 to reduce the functional and structural changes of diabetic nephropathy in male Akita (Ins2<sup>WT/C86Y</sup>) mice, a model of type 1 diabetes that is associated with the development of changes in the kidney that are similar to human diabetic nephropathy (18,26,40).

We observed early and sustained increases in the blood glucose concentrations in our Akita mice, as reported previously (18,26,40). Our major finding is that treatment with exogenous hrACE2 slows the progression of diabetic nephropathy. The Akita diabetic mice develop an increase in the urinary albumin excretion rate in association with renal and glomerular hypertrophy, mesangial matrix expansion, and an increase in GBM thickness compared with littermate nondiabetic mice. The increase in albumin excretion, an early functional abnormality in the natural history of nephropathy in patients with diabetes (41,42), was markedly reduced by hrACE2 treatment. ACE2 activity increased in the plasma of treated mice; plasma and renal ANG II levels declined whereas ANG 1–7 levels rose in the treated Akita mice. These observations are consistent with the hypothesis that ACE2 plays an important role in the processing of angiotensin peptides in the plasma and kidney (13,15,16) and that ANG II–dependent injury (via the AT1 receptors) in the diabetic kidney is accelerated by reduced ACE2 activity (18). Whether the changes in renal angiotensin peptide levels reflect the changes in plasma angiotensin levels and/or an active intrarenal process remains to be clarified. Consistent with previous studies (18,40), we observed mild hypertension in the diabetic Akita mice and hrACE2 treatment lowered blood pressure in association with the decrease in plasma ANG II levels, an effect that may contribute to renal protection.

Glomerular hypertrophy and mesangial matrix expansion, early features of human diabetic nephropathy, were reduced by hrACE2 treatment, confirming that modulation of angiotensin peptide metabolism and its downstream effects are involved in the development of diabetic nephropathy.
effects can attenuate diabetic kidney injury in the diabetic mouse. The RAS is activated in the diabetic milieu and increasing ACE2 activity may provide an alternate and important strategy to limit the role of the RAS in progressive diabetic nephropathy. Increased renal NADPH oxidase activity and activation of the PKC system are two canonical pathways known to play a fundamental role in the pathophysiology of diabetic nephropathy (28–30). Expression analysis of the renal cortical NADPH subunits revealed that both NOX2 (gp91phox) and p47 subunits were increased in diabetic Akita mice in agreement with previous findings in a type 1 diabetic model (29). Along with these changes in NADPH oxidase subunit expression, NADPH oxidase activity increased in the kidney cortex of our diabetic Akita mice. Importantly, hrACE2 treatment reduced renal cortical protein levels of PKCα and PKCβ1 and normalized NADPH oxidase subunit expression and activity in diabetic Akita mice.

We used an in vitro system of cultured primary rat mesangial cells to provide further insights into the mechanisms responsible for renoprotective effect of hrACE2 treatment in our diabetic Akita mice. Both high glucose concentrations and ANG II increased NADPH oxidase activity in vitro, and hrACE2 treatment attenuated high glucose– and ANG II–induced DHF staining and NADPH oxidase activation in the mesangial cells. Blockade of ANG 1–7 signaling with a Mas receptor peptide antagonist limited the protective effect of hrACE2 in vitro. Taken together with our finding that kidney cortical levels of ANG 1–7 were increased in the treated diabetic mice, these in vitro findings support the hypothesis that the protective effect of hrACE2 on diabetic injury was mediated, at least in part, by an increase in ANG 1–7 levels and attenuation of oxidative stress. Indeed, treatment with ANG 1–7 reduces renal NADPH oxidase activity and urinary albumin excretion in diabetic hypertensive rats (43), whereas the loss of ANG 1–7 receptor (Mas receptor) leads to glomerular hyperfiltration and albuminuria (44), changes that are characteristic of early diabetic nephropathy. Finally, our data also suggest that hrACE2-induced reduction in NADPH oxidase activity in vivo is due in part to the decrease in plasma and kidney ANG II levels.

In summary, we have shown that hrACE2 treatment improves kidney function and structure in a murine model of diabetic nephropathy. The ability of hrACE2 to suppress high glucose– and ANG II–induced activation of NADPH oxidase and to limit diabetic nephropathy is consistent with the notion that it functions as a negative regulator of the RAS. These beneficial effects of hrACE2 were not due to changes in plasma glucose levels, although there was a normalizing effect on blood pressure that may contribute to the renoprotection. Enhancing ACE2 activity may represent a novel therapeutic strategy to minimize the rate of progression of diabetic kidney disease. Additional work will be required to determine whether hrACE2 provides an incremental benefit over AT1 receptor blockade and/or ACE inhibition.

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Apeiron Biologics is a biotechnology company founded by J.M.P. H.L. owns stock in and is the chief executive officer for Apeiron Biologics. M.S. owns stock in and is the chief operating officer of Apeiron Biologics. E.J. owns stock in and is employed by Apeiron Biologics. J.M.P. owns stock in and is a member of the supervisory board for Apeiron Biologics. No other potential conflicts of interest relevant to this article were reported.

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REFERENCES

1. Levey AS, Atkins R, Coresh J, Cohen EP, Collins AJ, Eckardt KU, Nahas ME, Jaber BL, Jadoul M, Levin A, Powe NR, Rossert J, Wheeler DC, Lampeij N, Eknoyan G. Chronic kidney disease as a global public health problem: approaches and initiatives—a position statement from Kidney Disease: Improving Global Outcomes. Kidney Int 2007;72:247–259

2. Levey AS, Andreoli SP, DuBoise T, Provenzano R, Collins AJ. Chronic kidney disease: common, harmful, and treatable—World Kidney Day 2007. J Am Soc Nephrol 2007;18:374–378

3. Coresh J, Selvin E, Stevens LA, Manzi J, Kusek JW, Eggers P, Van Lente F, Levey AS. Prevalence of chronic kidney disease in the United States. JAMA 2007;298:2038–2047

4. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD. The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy: the Collaborative Study Group. N Engl J Med 1993;329:1456–1462

5. Cooper ME. Pathogenesis, prevention, and treatment of diabetic nephropathy. Lancet 1998;352:213–216

6. Lewis EJ, Hunsicker LG, Clarke WR, Berl T, Pohl MA, Lewis JB, Ritz E, Atkins RC, Rohde R, Raz I, Collaborative Study Group. Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. N Engl J Med 2001;345:851–860

7. Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving HH, Remuzzi G, Snapinn SM, Zhang Z, Shabihana S, RENAAL Study Investigators. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. N Engl J Med 2001;345:861–869

8. Huang W, Gallois Y, Bouby N, Bruneval P, Heudes D, Belair MF, Krege JH, Meneton P, Marre M, Smithies O, Alhenc-Gelas F. Genetically increased angiotensin I-converting enzyme level and renal complications in the diabetic mouse. Proc Natl Acad Sci U S A 2001;98:13330–13334

9. Takamura SR, Hooper NM, Ryall R, Karran E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme: cloning and functional expression as a captorpril-insensitive carboxypeptidase. J Biol Chem 2000;275:33238–33243

10. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagnino N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin I–9. Circ Res 2000;87:E1–E9

11. Oudit GY, Crackower MA, Backx PH, Penninger JM. The role of ACE2 in cardiovascular physiology. Trends Cardiovasc Med 2003;13:93–101

12. Hamming I, Cooper ME, Haagmans BL, Hooper NM, Konstanje R, Osterhaus AD, Timens W, Turner AJ, Navis G, van Goor H. The emerging role of ACE2 in physiology and disease. J Pathol 2007;212:1–11

13. Chappell MC, Modragll JG, Diaz DF, Ferrario CM. Novel aspects of the renal renin-angiotensin system: angiotensin (1–7), ACE2 and blood pressure regulation. Contrib Nephrol 2004;143:77–89

14. Ye M, Wysocki J, Naaz P, Salabat MR, LalPointe MS, Batlle D. Increased ACE 2 and decreased ACE protein in renal tubules from diabetic mice: a renoprotective combination? Hypertension 2004;43:1120–1125

15. Wysocki J, Ye M, Soler MJ, Gurley SB, Xiao HD, Bernstein KE, Goffman TM, Chen S, Batlle D. ACE and ACE2 activity in diabetic mice. Diabetes 2006;55:2192–2199

16. Tikelis C, Johnston CI, Forbes JM, Burns WC, Burrell LM, Risvanis J, Cooper ME. Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. Hypertension 2003;41:392–397

17. Oudit GY, Herzenberg AM, Kassiri Z, Wong D, Reich H, Khokha R,
Crackower MA, Backx PH, Penninger JM, Scholey JW. Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis. Am J Pathol 2006;168:1808–1820

18. Wong DW, Oudit GY, Reich H, Kassiri Z, Zhou J, Liu QC, Backx PH, Penninger JM, Herzenberg AM, Scholey JW. Loss of angiotensin-converting enzyme-2 (Ace2) accelerates diabetic kidney injury. Am J Pathol 2007;171:438–451

19. Mizuiri S, Hemmi H, Arita M, Ohashi Y, Tanaka Y, Miyagi M, Sakai K, Ishikawa Y, Shibuya K, Hase H, Aikawa A. Expression of ACE and ACE2 in individuals with diabetic kidney disease and healthy controls. Am J Kidney Dis 2008;51:613–623

20. Reich HN, Oudit GY, Penninger JM, Scholey JW, Herzenberg AM. Decreased glomerular and tubular expression of ACE2 in patients with type 2 diabetes and kidney disease. Kidney Int 2008;74:1610–1616

21. Soler MJ, Barrios C, Oliva R, Batlle D. Pharmacologic modulation of ACE2 expression. Curr Hypertens Rep 2008;10:410–414

22. Oudit GY, Imai Y, Kuba K, Scholey JW, Penninger JM. The role of ACE2 in pulmonary diseases—relevance for the nephrologist. Nephrol Dial Transplant 2009;24:1362–1365

23. Huang L, Sexton DJ, Skogerson K, Devlin M, Smith R, Sanyal I, Parry T, Kent R, Enright J, Wu QL, Conley G, De Oliveira D, Morganelli L, Ducar M, Wescott CR, Ladner RC. Novel peptide inhibitors of angiotensin-converting enzyme 2. J Biol Chem 2003;278:15522–15540

24. Redling S, Pfaff IL, Leitges M, Vallon V. Immunolocalization of protein kinase C isoenzymes alpha, beta I, beta II, delta, and epsilon in mouse kidney. Am J Physiol Renal Physiol 2004;287:F289–F298

25. Kassiri Z, Oudit GY, Kandalam V, Awad A, Wang X, Ziou X, Maeda N, Herzenberg AM, Scholey JW. Loss of TIMP3 enhances interstitial nephritis and fibrosis. J Am Soc Nephrol 2000;10:1223–1235

26. Kakoki M, Takahashi N, Jennette JC, Smithies O. Diabetic nephropathy is markedly enhanced in mice lacking the bradykinin B2 receptor. Proc Natl Acad Sci USA 2004;101:13302–13305

27. Tesch GH. MCP-1/CCL2: a new diagnostic marker and therapeutic target for progressive renal injury in diabetic nephropathy. Am J Physiol Renal Physiol 2008;294:F697–F701

28. Kelly DJ, Zhang Y, Hepper C, Gow RM, Jaworski K, Kemp BE, Wilkinson-Templeton GH. Immunolocalization of protein kinase C isoenzymes alpha, beta I, beta II, delta, and epsilon in mouse kidney. Am J Physiol Renal Physiol 2004;286:F1039–F1045

29. Cristovam PC, Arnoni CP, de Andrade MC, Casarini DE, Pereira LG, Schor N, Boim MA. ACE-dependent and chymase-dependent angiotensin II generation in normal and glucose-stimulated human mesangial cells. Exp Biol Med (Maywood) 2008;233:1035–1043

30. Su Z, Zimpelmann J, Burns KD. Angiotensin-(1–7) inhibits angiotensin II-stimulated phosphorylation of MAP kinases in proximal tubular cells. Kidney Int 2006;69:2212–2218

31. Suissa S, Hutchinson T, Brophy JM, Kezouh A. ACE-inhibitor use and the long-term risk of renal failure in diabetes. Kidney Int 2006;69:913–919

32. Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients: the Heart Outcomes Prevention Evaluation Study Investigators. N Engl J Med 2000;342:145–153

33. Turner AJ, Hiscox JA, Hooper NM. ACE2: from vasopeptidase to SARS coronavirus receptor. Trends Pharmacol Sci 2004;25:291–294

34. Soler MJ, Wysocki J, Ye M, Lloveras J, Kanwar Y, Batlle D. ACE2 inhibition worsens glomerular injury in association with increased ACE expression in streptozotocin-induced diabetic mice. Kidney Int 2007;72:614–623

35. Tikellis C, Bialkowski K, Petre J, Sheehy K, Su Q, Johnston C, Cooper ME, Thomas MC. ACE2 deficiency modifies renoprotection afforded by ACE inhibition in experimental diabetes. Diabetes 2008;57:1018–1025

36. Gurley SB, Clare SE, Snow KP, Hu A, Meyer TW, Coffman TM. Impact of genetic background on nephropathy in diabetic mice. Am J Physiol Renal Physiol 2006;290:F214–F222

37. Garstein HC, Mann JF, Yi Q, Zinnman B, Dinneen SF, Halle JP, Young J, Rashkow A, Joyce C, Nawaz S, Yusuf S, HOPE Study Investigators. Albuminuria and risk of cardiovascular events, death, and heart failure in diabetic and nondiabetic individuals. JAMA 2001;286:421–426

38. Perkins BA, Ficociello LH, Silva KH, Finkelstein DM, Warram JH, Krolewski AS. Regression of microalbuminuria in type 1 diabetes. N Engl J Med 2003;348:2285–2293

39. Gerstein HC, Mann JF, Yi Q, Zinnman B, Dinneen SF, Hoogwerf B, Hallé JP, Young J, Rashkow A, Joyce C, Nawaz S, Yusuf S, HOPE Study Investigators. Regression of microalbuminuria in type 1 diabetes. N Engl J Med 2003;348:2285–2293

40. Perkerson L, Zimpelmann J, Burns KD. Angiotensin-(1–7) prevents activation of NADPH oxidase and renal vascular dysfunction in diabetic hypertensive rats. Am J Physiol 2008;298:F25–33

41. Pinheiro SV, Ferreira AJ, Kitten GT, da Silveira KD, da Silva DA, Santos SH, Gava E, Castro CH, Magalhães JA, da Mota RK, Botelho-Santos GA, Bader M, Alenina N, Santos RA, Simoes e Silva AC. Genetic deletion of the angiotensin-(1–7) receptor Mas leads to glomerular hyperfiltration and microalbuminuria. Kidney Int 2009;75:1184–1190