ACE2 Deficiency Modifies Renoprotection Afforded by ACE Inhibition in Experimental Diabetes

Chris Tikellis, Katarzyna Bialkowski, Josepha Pete, Karen Sheehy, Qui Su, Colin Johnston, Mark E. Cooper, and Merlin C. Thomas

OBJECTIVE—The degradation of angiotensin (Ang) II by ACE2, leading to the formation of Ang 1–7, is an important step in the renin-angiotensin system (RAS) and one that is significantly altered in the diabetic kidney. This study examines the role of ACE2 in early renal changes associated with diabetes and the influence of ACE2 deficiency on ACE inhibitor–mediated renoprotection.

RESEARCH DESIGN AND METHODS—Diabetes was induced by streptozotocin in male c57bl6 mice and ACE2 knockout (KO) mice. After 5 weeks of study, animals were randomized to receive the ACE inhibitor perindopril (2 mg · kg^{-1} · day^{-1}). Wild-type mice were further randomized to receive the selective ACE2 inhibitor MLN-4760 (10 mg · kg^{-1} · day^{-1}) and followed for an additional 5 weeks. Markers of renal function and injury were then assessed.

RESULTS—Induction of diabetes in wild-type mice was associated with a reduction in renal ACE2 expression and decreased Ang 1–7. In diabetic mice receiving MLN-4760 and in ACE2 KO mice, diabetes-associated albuminuria was enhanced, associated with an increase in blood pressure. However, renal hypertrophy and fibrogenesis were reduced in diabetic mice with ACE2 deficiency, and hyperfiltration was attenuated. Diabetic wild-type mice treated with an ACE inhibitor experienced a reduction in albuminuria and blood pressure. These responses were attenuated in both diabetic ACE2 KO mice and diabetic mice receiving MLN-4760. However, other renoprotective and antifibrotic actions of ACE inhibition in diabetes were preserved in ACE2-deficient mice.

CONCLUSIONS—The expression of ACE2 is significantly modified by diabetes, which impacts both pathogenesis of kidney disease and responsiveness to RAS blockade. These data indicate that ACE2 is a complex and site-specific modulator of diabetic kidney disease. ***Diabetes*** **57:1018–1025, 2008**

From the Juvenile Diabetes Research Foundation Danielle Alberti Memorial Centre for Diabetes Complications, Baker Medical Research Institute, Melbourne, Australia.

Address correspondence and reprint requests to Prof. Merlin Thomas, Baker Medical Research Institute, P.O. Box 6492, St. Kilda Central, Melbourne 8008, Australia. E-mail: merlin.thomas@baker.edu.au.

Received for publication 29 August 2007 and accepted in revised form 17 January 2008.

Published ahead of print at http://diabetes.diabetesjournals.org on 30 January 2008. DOI: 10.2337/db07-1212.

Additional information for this article can be found in an online appendix at http://dx.doi.org/10.2337/db07-1212.

AER, albumin excretion rate; Ang, angiotensin; AT_1, type 1 angiotensin; RAS, renin-angiotensin system.

Blockade of the renin-angiotensin system (RAS) is considered to be among the most important interventions for the prevention and management of diabetic kidney disease (1). Drugs that inhibit ACE or block activation of the type 1 angiotensin (AT_1) receptor are effective in attenuating renal damage in diabetes over and above any reduction in systemic blood pressure (2,3). Historically, these actions have been linked to reduced signaling through angiotensin (Ang) II–dependent pathways. However, at least some the systemic effects of RAS blockade may be mediated by Ang 1–7, a potent vasodilator, with actions that antagonize or compensate those of Ang II (4). In the kidney, Ang 1–7 is largely derived from the degradation of Ang II by the zinc-dependent carboxypeptidase ACE2 (Fig. 1) (5). We and others, have previously shown that the pattern of ACE2 expression in the kidney is significantly modified following the induction of diabetes in experimental animals (6–10). Despite this, the potential role of ACE2 in modulating the efficacy of ACE inhibition has not been previously considered. This study specifically examines the role of ACE2 in early renal changes associated with diabetes, using two different models of ACE2 deficiency: *ACE2* KO mice and wild-type mice treated with a selective ACE2 inhibitor (8). In addition, this study examines the potential influence of ACE2 deficiency on ACE inhibitor–mediated renoprotection in experimental diabetes.

RESEARCH DESIGN AND METHODS

Induction of experimental type 1 diabetes. Wild-type male c57bl6 mice and *ACE2* KO mice (donated by Dr. Penninger, Institute for Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria [11]), aged 10 weeks, were randomly allocated to receive streptozotocin (55 mg/kg, Sigma Chemical, St. Louis, MO) or buffer (sodium citrate buffer pH 4.5) delivered in five consecutive daily doses. This regimen induces an insulinopenic form of diabetes associated with hyperglycaemia (blood glucose ~30 mmol/l) but with sufficient β-cell reserve to prevent ketosis or require insulin supplementation. The presence of diabetes was confirmed by a blood glucose level >15 mmol/l 1 week after the first dose of streptozotocin. Throughout the study, animals were given access to food and water ad libitum.

Treatment arms. Control and diabetic c57bl6 and ACE2 KO mice, after 5 weeks without treatment, were randomized to receive treatment with the ACE inhibitor perindopril (Servier, Neuilly, France) at a dose of 2 mg · kg^{-1} · day^{-1} in drinking water or no treatment for 5 weeks. This delayed intervention was chosen to mimic the clinical scenario of introducing ACE inhibition in patients with established microalbuminuria. In addition, previous experimental studies have demonstrated that, when delivered from the onset of diabetes, ACE inhibition is completely effective in preventing diabetes-associated increases in albuminuria. Wild-type mice were further randomized to receive the specific nonpeptide ACE2 inhibitor MLN-4760 (Millennium Pharmaceuticals, Cambridge, Boston, MA) at a dose of 10 mg · kg^{-1} · day^{-1} in drinking water or no treatment for 5 weeks after 5 weeks diabetes duration. Each group contained 10 animals. A period of 10 weeks diabetes duration was chosen specifically to examine the effects of ACE2 on early physiological changes in the diabetic kidney, including albuminuria, hypertrophy, and hyperfiltration,

FIG. 1. Mediators of angiotensin peptide synthesis and degradation. AP-A/N, aminopeptidase A and aminopeptidase N; CAGE, chymostatin-sensitive angiotensin II-generating enzyme; NEP, neprilysin; PEP, prolyl endopeptidase.

TABLE 1
General assessment parameters in each of the study groups

	Body weight (g)	Glucose (mmol/l)	Glycated Hb (%)	Systolic blood pressure (mmHg)	Albumin excretion (μg/day)	Renal mass (g/m^2)	Creatinine clearance (ml/min)	ERPF (ml/min)
c57 (wild type)	33 ± 1	11.1 ± 1.6	4.1 ± 0.1	121 ± 1	47 ×/÷ 1.1	33 ± 1	0.26 ± 0.02	0.7 ± 0.1
c57 + ACEi	32 ± 1	10.5 ± 1.2	4.1 ± 0.1	113 ± 1*	30 ×/÷ 1.1*	34 ± 1	0.27 ± 0.04	0.5 ± 0.1
c57 + ACE2i	33 ± 1	8.0 ± 1.0*	3.8 ± 0.2	120 ± 2	30 ×/÷ 1.1*	35 ± 1	0.34 ± 0.02	1.4 ± 0.2
c57 + ACEi + ACE2i	33 ± 1	8.4 ± 1.3*	3.6 ± 0.1*	118 ± 1	39 ×/÷ 1.1	36 ± 1	0.32 ± 0.03	1.3 ± 0.1
Diabetes + c57	26 ± 1*	30 ± 1.3*	12.0 ± 0.6*	119 ± 2	218 ×/÷ 1.1*	46 ± 1*	0.66 ± 0.01*	2.1 ± 0.2*
Diabetes + c57 + ACEi	27 ± 2*	27 ± 1.4*	13.5 ± 0.6	113 ± 1*†	102 ×/÷ 1.1*†	45 ± 1*	0.66 ± 0.02*	1.5 ± 0.1*
Diabetes + c57 + ACE2i	28 ± 2*	29 ± 1.2*	13.7 ± 0.7*	125 ± 2†	302 ×/÷ 1.1*†	42 ± 1*†	0.37 ± 0.03*†	1.1 ± 0.1*†
Diabetes + c57 + ACEi + ACE2i	26 ± 2 *	28 ± 1.3*	12.9 ± 0.6*	125 ± 1*†	294 ×/÷ 1.3*†	40 ± 1*†	0.28 ± 0.03†	1.2 ± 0.1*†
ACE2 KO	31 ± 1*	9.1 ± 1.1*	3.5 ± 0.2*	122 ± 2	35 ×/÷ 1.1*	34 ± 1	0.33 ± 0.02	1.3 ± 0.2*
ACE2 KO + ACEi	30 ± 1*	9.0 ± 1.3*	4.0 ± 0.1	120 ± 3	39 ×/÷ 1.1	32 ± 1	0.27 ± 0.04‡	1.1 ± 0.1*
Diabetes + *ACE2* KO	24 ± 1*‡	29.1 ± 1*‡	11.9 ± 0.6*‡	126 ± 2*†‡	193 ×/÷ 1.1*‡	43 ± 1*†‡	0.45 ± 0.03*†‡	1.2 ± 0.1*†
Diabetes + *ACE2* KO +ACEi	25 ± 1*‡	28.5 ± 1*‡	13.9 ± 0.4*‡	135 ± 4*†‡	255 ×/÷ 1.1*†‡	36 ± 1†§	0.32 ± 0.03†§	0.8 ± 0.1‡§

Data are means ± SEM except for AER, which is shown as its geometric mean ×/÷ 1.1 tolerance factor. $P < 0.05$ *vs. control c57, †vs. diabetic c57, ‡vs. control *ACE2* KO, and §vs. diabetic *ACE2* KO. ERPF, effective renal plasma flow.

without the potentially confounding effects of cardiac dysfunction or glomerulosclerosis that have been described in older male ACE2 KO mice (11,12). However, it should be appreciated that 10 weeks diabetes duration in mice bred on a c57bl6 background is insufficient to produce significant interstitial fibrosis or mesangial changes, structural features associated with more advanced nephropathy, as previously described in other rodent models (13).

General assessment parameters.

The following parameters were serially measured: body weight; blood glucose, measured using a glucometer (Accutrend; Boehringer Mannheim, Biochemica, Mannheim, Germany); systolic blood pressure, measured by tail cuff plethysmography in conscious, prewarmed mice (14); and glycated hemoglobin, measured by high-performance liquid chromatography (HPLC) (CLC330 GHb Analyzer; Primus, Kansas City, MO) (15). Urine was collected from animals placed in individual metabolic cages (Iffa Credo, L'Arbresele, France) for 24 h, allowing measurement of the urinary excretion of mouse albumin by enzyme-linked immunosorbent assay (Bethyl Laboratories, Montgomery, TX). Creatinine clearance was estimated from serum and urine creatinine concentrations, as determined by HPLC according to ADMCC guidelines (16). Effective renal plasma flow was estimated from the clearance of endogenous hippuric acid clearance in conscious animals and determined by HPLC as previously described (17). After 10 weeks of experimental diabetes, mice were culled by a fatal overdose of anesthetic followed by cardiac exsanguination. The kidneys were rapidly dissected out, weighed, and bisected, with one-half snap-frozen in liquid nitrogen and stored at −80°C before use and the other half embedded in paraffin.

Activity of the intrarenal RAS. Gene expression of various components of the RAS, including *ACE*, *ACE2* and prorenin mRNA in cortical homogenates, were assessed by real-time quantitative RT-PCR. This was performed using the TaqMan system based on real-time detection of accumulated fluorescence

(ABI Prism 7700; Perkin-Elmer, PE Biosystems, Foster City, CA) as previously utilized by our group (18). Gene expression was normalized to 18S mRNA and reported as ratios compared with the level of expression in untreated control mice, which were given the arbitrary value of 1.

Renal and serum ACE2 activity was determined following incubation with the intramolecularly quenched synthetic ACE2-specific substrate *o*-aminobezoic acid-Ser-Pro-Tyr(NO_2)-OH (Peptides International). Briefly, 5 μl heparinized plasma or cortical homogenate was added to wells containing 95 μl of 100 mmol/l Tris pH 6.5, 1.5 mol/l NaCl, 1 mmol/l PMSF, 1 mmol/l N-ethylmaleimide, 2 μg bovine serum albumin, and 33 μmol/l substrate. Reactions were in triplicates (one of each three wells constituted a blank). Blank wells contained the same components, but 1 mmol/l EDTA was also added. This inhibited activity to the same extent as MLN-4760 (data not shown). After incubation at 37°C for 1–18 h, fluorescence was measured using an F-max plate fluorescence reader with an excitation of 320 nm and an emission of 420 nm. Total fluorescence was corrected for volume (in plasma samples) or protein content (in tissue homogenates) after subtracting blank values. Purified recombinant ACE2 was used to calibrate activity, and results are expressed as nanogram equivalents. Renal and serum ACE activity was determined following incubation with the intramolecularly quenched synthetic ACE-specific substrate *o*-aminobezoic acid-FRK(Dnp)-P (Peptides International). Results are expressed as a percentage of activity observed in control samples. Renal ACE2 expression was further localized following immunohistochemical staining of paraffin-fixed kidney sections using a rabbit polyclonal anti-ACE2 antibody diluted 1:500 raised against ACE2 residues 489–508 (antibody donated by Millennium Pharmaceuticals).

The intrarenal concentration of Ang 1–7 was measured on snap-frozen renal cortical samples by a commercial radioimmunoassay (ProSearch International, Malvern, Australia) utilizing an Ang 1–7 selective polyclonal antibody and ^{125}I–Ang 1–7. Before analysis, samples were homogenized in ice-cold methanol in the presence of protease inhibitors (50 mmol/l ethylenediaminetetraacetic acid, 0.5 mmol/l *o*-phenanthroline, 1 mmol/l N-ethylmalemide, and 0.1 mmol/l pepstatin A). Samples were then centrifuged to remove protein, and the upper (aqueous) phase was removed and lyophilized in glass tubes at −80°C. Results are expressed adjusted for the wet weight of the sample.

Markers of renal hypertrophy and nephropathy. Each kidney was cleaned of fat and weighed with the result expressed as the combined weight adjusted for body surface area (grams per meters squared). The expression of early markers of nephropathy and matrix accumulation in diabetes, including osteopontin and fibronectin, was determined by real-time quantitative RT-PCR in cortical homogenates, as previously described (19). The glomerular expression of fibronectin protein was further quantified by a computerized image analysis of sections stained with a polyclonal rabbit anti-human fibronectin (DaKO, Glostrop, Denmark) antibody and detected using a secondary biotinylated goat anti-rabbit antibody and the ABC detection system (Vector Laboratories, Burlingame, CA).

Statistical analysis. Continuous data are expressed as means ± SEM except for AER, which is expressed as its geometric mean (×/÷ tolerance factor) and analyzed after log transformation. Differences in the mean among groups were compared using two-way ANOVA with diabetes status and ACE2 status as the two variables. Pairwise multiple comparisons were made with the Student-Newman-Keuls post hoc analysis to detect significant differences between groups. $P < 0.05$ was considered statistically significant.

RESULTS

General parameters. The induction of diabetes by streptozotocin in both c57bl6 and *ACE2* KO mice was associated with a significant increase in plasma glucose concentration (~25–30 mmol/l) and glycated hemoglobin (12–15%) compared with respective nondiabetic controls (Table 1). Plasma glucose levels in diabetic animals were unaffected by the inhibition of ACE, ACE2, or their combination, while in nondiabetic mice, there was a small reduction in fasting glucose levels in ACE2 deficient mice. Consistent with the initial reports in *ACE2* KO mice (11), tail cuff systolic blood pressure levels in the absence of diabetes were not significantly different between c57bl6 and *ACE2* KO mice or c57bl6 mice treated with an ACE2 inhibitor. However, following induction of diabetes, systolic blood pressure rose significantly in both *ACE2* KO mice and wild-type mice treated with a selective ACE2 inhibitor compared with untreated c57bl6 mice with diabetes. In c57bl6 mice, systolic blood pressure was reduced by the ACE inhibitor perindopril both in the presence and absence of diabetes. However, this hypotensive action was attenuated in *ACE2* KO and c57bl6 mice receiving an ACE2 inhibitor in combination with perindopril (Table 1).

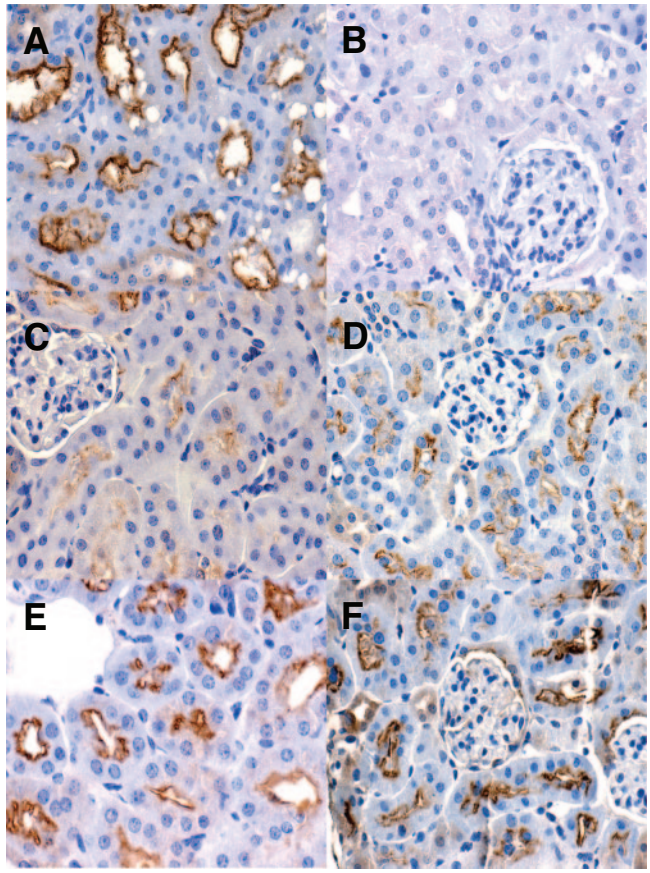

FIG. 2. Immunohistochemistry staining for ACE2 expression in cortical sections. Original magnification ×850. *A*) c57bl6; *B*) *ACE2* KO; *C*) c57bl6 + diabetes; *D*) c57bl6 + diabetes + perindopril; *E*) c57bl6 + diabetes + MLN-4760; *F*) c57bl6 + diabetes + perindopril + MLN-4760. (Please see http://dx.doi.org/10.2337/db07-1212 for a high-quality digital representation of this figure.)

Markers and mediators of RAS. The induction of diabetes in c57bl6 mice led to a reduction in the cortical expression of *ACE2* mRNA (Fig. 3*A*) and ACE2 protein, particularly at the apical surface of the S1–2 proximal tubules (Fig. 2). This was associated with a significant reduction in cortical levels of Ang 1–7 (Fig. 4). Despite this, renal ACE2 activity, measured in cortical homogenates, paradoxically increased following induction of diabetes in c57bl6 mice (Fig. 3*B*). In addition, induction of diabetes in c57Bl6 mice was associated with a greater than twofold increase in plasma ACE2 activity (Fig. 3*C*).

Treatment of c57Bl6 mice with an ACE2 inhibitor reduced plasma ACE2 activity by 75–80% in both control and diabetic animals (Fig. 3*C*). MLN-4760 had no detectable effect on cortical ACE2 activity in control animals but reduced cortical ACE2 activity in diabetic mice to levels observed in untreated control animals (Fig. 3*B*). In addition, treatment with MLN-4760 was also associated with reduced cortical levels of Ang 1–7 in both control and diabetic mice (Fig. 4). *ACE2* KO mice had no detectable *ACE2* gene expression or activity in renal or plasma samples. *ACE2* KO mice also demonstrated reduced renal

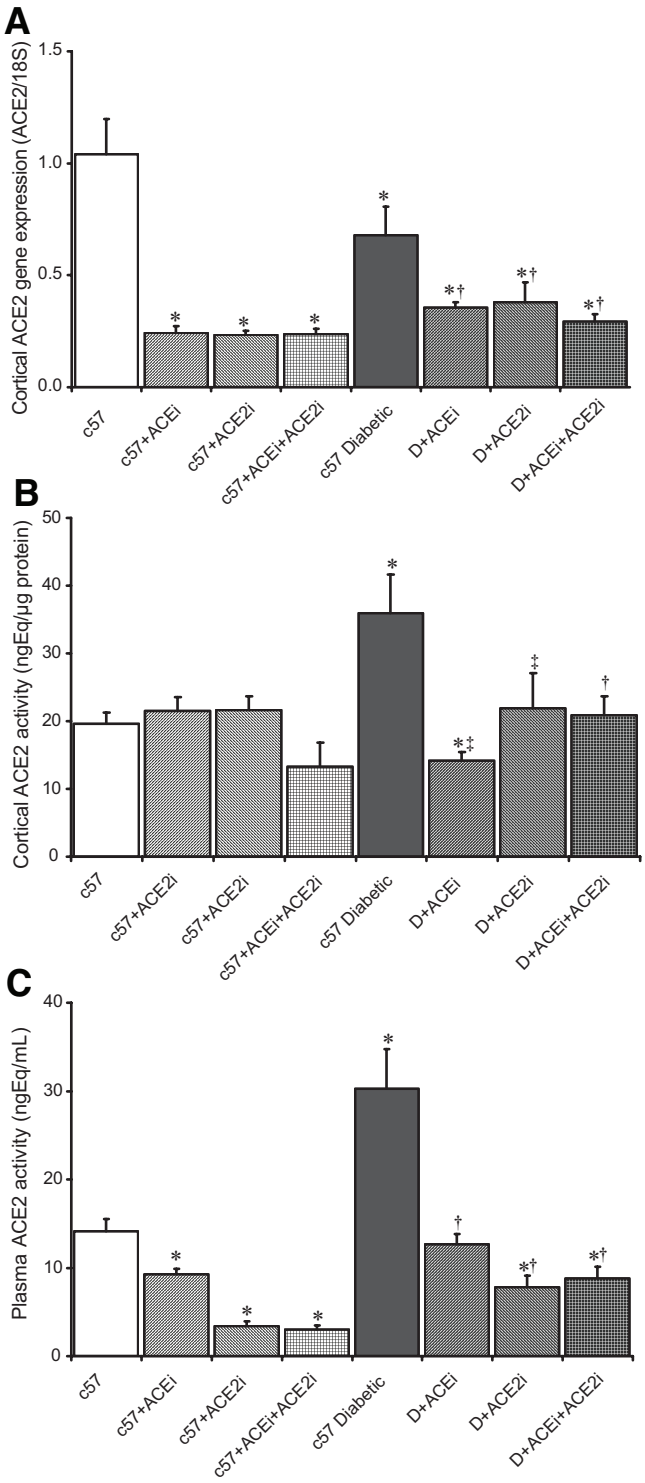

FIG. 3. Cortical *ACE2* gene expression (*A*), ACE2 activity in cortical homogenates (*B*), and ACE2 activity in heparinized plasma (*C*). No ACE2 expression activity was detected in ACE2 KO mice (data not shown). Data are shown as means ± SEM. $P < 0.05$ *vs. control c57 and †vs. diabetic c57.

cortical levels of Ang 1–7 compared with those in wild-type animals (Fig. 4).

The ACE inhibitor perindopril reduced the expression and activity of ACE in both plasma and in the kidney by 50–60% (Fig. 5). The magnitude of this effect was similar in both control and diabetic animals and in c57bl6 mice and *ACE2* KO mice. Although the ACE2 enzyme is not

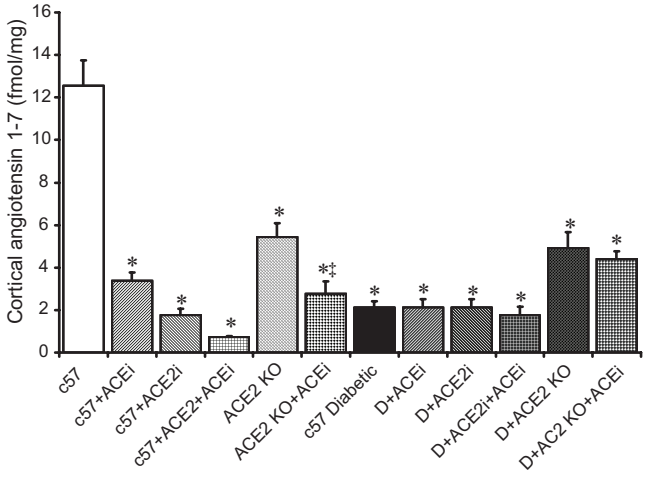

FIG. 4. Angiotensin 1–7 concentration in cortical homogenates. Data are means ± SEM. $P < 0.05$ *vs. control c57 and ‡vs. control ACE2 KO.

inhibited by ACE inhibitors in vitro, perindopril also significantly modified the expression and activity of ACE2 in our models. Treatment of c57Bl6 mice with perindopril reduced plasma ACE2 activity and cortical ACE2 activity in both control and diabetic animals (Fig. 3*C*). This was also associated with a significant reduction in cortical levels of Ang 1–7 when compared with those of untreated wild-type animals (Fig. 4).

Conversely, the selective ACE2 inhibitor MLN-4760 also reduced the renal expression and activity of ACE in both control and diabetic animals. This action was not due to direct inhibition of ACE, as the addition of equivalent concentrations of MLN-4760 to control plasma did not inhibit ACE, and the mixture of plasma from animals receiving an ACE2 inhibitor did not cause additional inhibition of ACE (data not shown).

The induction of experimental diabetes in c57bl6 mice was also associated with an increase in the intrarenal expression of prorenin mRNA (2.3 ± 0.5-fold), as previously described by other groups (20). However, in both diabetic *ACE2* KO mice (0.3 ± 0.1-fold) and diabetic mice receiving an ACE2 inhibitor (0.4 ± 0.1-fold), no increase was demonstrated. Treatment with an ACE inhibitor also increased prorenin expression in the kidney as a known part of its negative feedback loop (c57bl6 plus perindopril, 5.3 ± 0.9-fold) (21). This increase was similar in both control and diabetic animals and unaffected in both *ACE2* KO mice (4.7 ± 0.7-fold) and mice receiving an ACE2 inhibitor (4.2 ± 0.4-fold).

Diabetes-associated albuminuria. The induction of diabetes in c57bl6 mice was associated with a 3.6-fold increase in urinary albumin excretion rate (AER) compared with that in untreated c57bl6 mice. Diabetic *ACE2* KO experienced a 5.2-fold increase in urinary AER compared with that in untreated *ACE2* KO mice (Table 1). When compared with baseline urinary albumin excretion levels, this increase in AER was statistically greater in *ACE2* KO mice ($P = 0.03$). As previously described (7,9), treatment with an ACE2 inhibitor alone significantly increased urinary AER in diabetic c57bl6 mice (Table 1). Treatment with an ACE inhibitor alone significantly reduced urinary AER in diabetic c57bl6 mice, consistent with its known antiproteinuric actions. However, this antiproteinuric effect of ACE inhibition was lost in diabetic

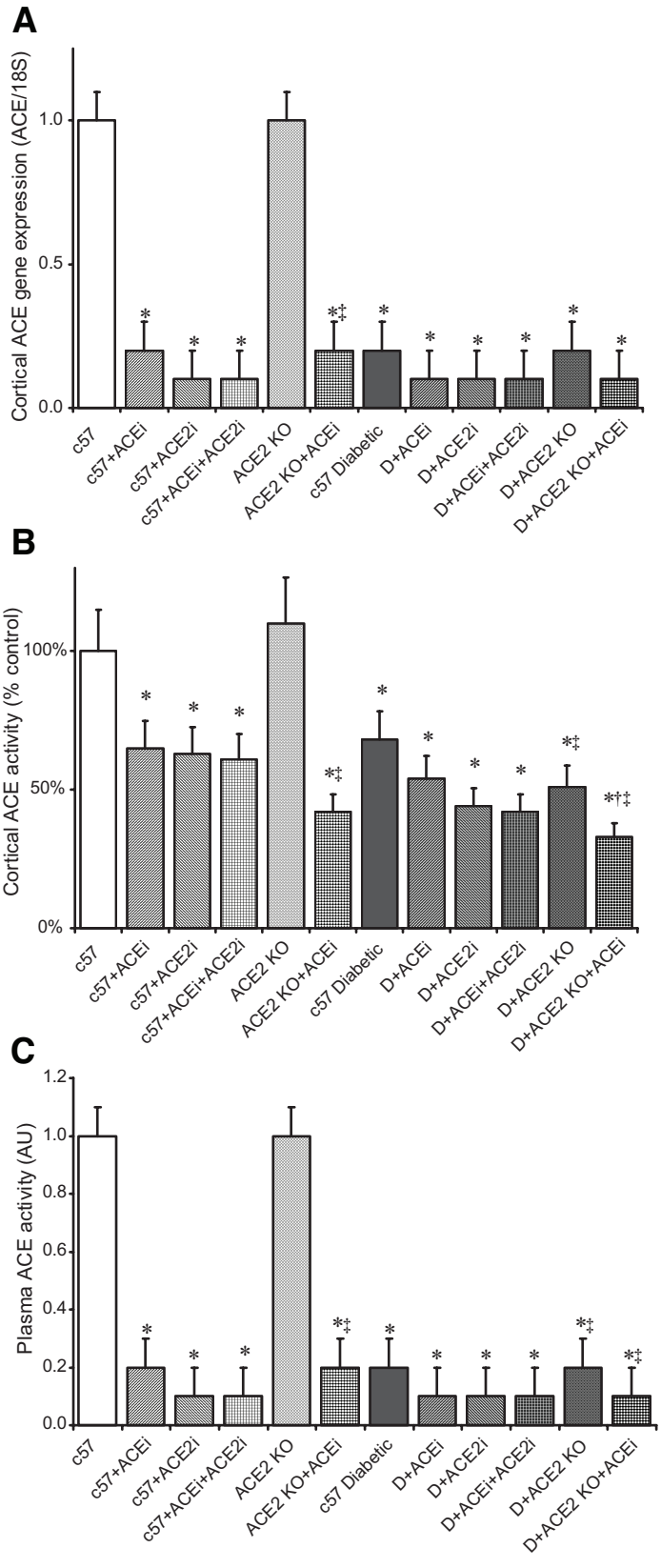

FIG. 5. Cortical *ACE* gene expression (*A*), ACE activity in cortical homogenates (*B*), and ACE activity in heparinized plasma (*C*). Data are means ± SEM. $P < 0.05$ *vs. control c57, †vs. diabetic c57, and ‡vs. control ACE2 KO.

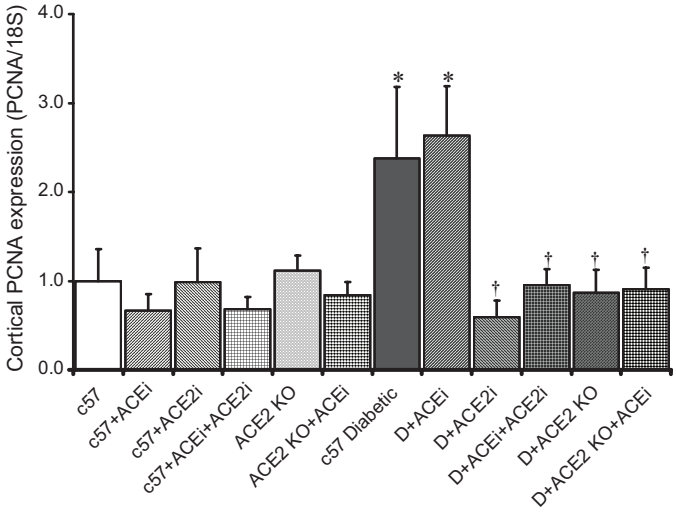

FIG. 6. The expression of the proliferating cell nuclear antigen in cortical homogenates, as measured by real-time RT-PCR. Data are means ± SEM. $P < 0.05$ *vs. control c57 and †vs. diabetic c57.

animals treated with a selective ACE2 inhibitor and in diabetic *ACE2* KO mice (Table 1).

Renal function in diabetic mice. Induction of diabetes in c57bl6 mice was associated with a significant increase in creatinine clearance and effective renal plasma flow compared with that in nondiabetic mice (Table 1). These increases were attenuated in diabetic mice treated with an ACE2 inhibitor and in diabetic *ACE2* KO mice. Creatinine clearance and effective renal plasma flow were not affected by ACE inhibition in wild-type animals with or without diabetes. However, in ACE2 KO mice, ACE inhibition significantly reduced creatinine clearance (Table 1).

Renal hypertrophy in diabetic mice. Induction of diabetes in c57bl6 mice was associated with a 45% increase in body weight–adjusted renal mass (Table 1). Induction of diabetes in *ACE2* KO mice was associated with an attenuated (27%) increase in renal mass. Similarly, treatment with a selective ACE2 inhibitor attenuated diabetes-associated renal hypertrophy. Reduced renal mass in ACE2 deficient mice was also associated with reduced cortical expression of proliferating cell nuclear antigen in renal samples compared with untreated diabetic c57bl6 mice (Fig. 6). Treatment with an ACE inhibitor alone had no effect on diabetes-associated renal hypertrophy or the expression of proliferating cell nuclear antigen in wild-type mice (Fig. 6). However, the use of ACE inhibitors in diabetic *ACE2* KO mice achieved a significant additional reduction in diabetes-associated renal hypertrophy.

Markers of diabetic nephropathy. As noted above, 10 weeks of experimental diabetes is insufficient to produce significant glomerulosclerosis or interstitial or mesangial expansion in mice bred on a c57bl6 background. However, increased cortical gene expression of the extracellular matrix protein fibronectin was observed following induction of diabetes (Fig. 7*A*), together with the accumulation of fibronectin protein in diabetic glomeruli (Fig. 7*B*). Treatment with a selective ACE2 inhibitor alone also reduced the expression of fibronectin in diabetic c57bl6 mice. Similarly, diabetic *ACE2* KO mice showed reduced accumulation of glomerular fibronectin compared with that in wild-type controls (Fig. 7*A*). Treatment with an ACE inhibitor significantly reduced the expression and accumulation of fibronectin in c57bl6 animals with diabe-

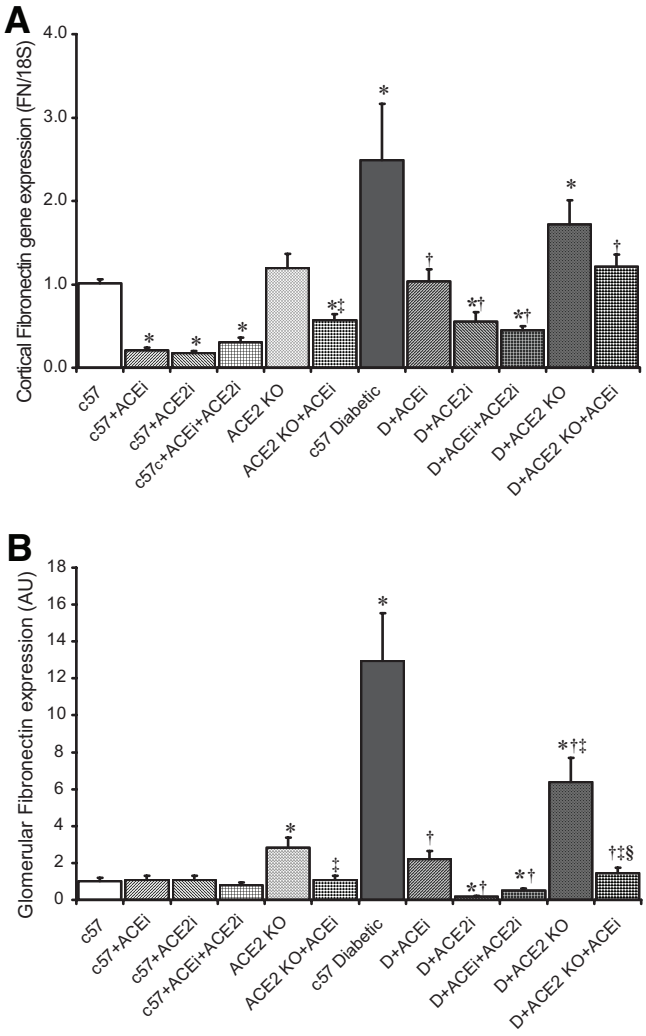

FIG. 7. The gene expression of fibronectin (*A*) in cortical homogenates, as measured by real-time RT-PCR, and the glomerular expression of fibronectin protein (*B*), as assessed by semiquantitative immunohistochemistry. Data are shown as means ± SEM. $P < 0.05$ *vs. control c57, †vs. diabetic c57, ‡ vs. control ACE 2KO, and §vs. diabetic ACE 2KO.

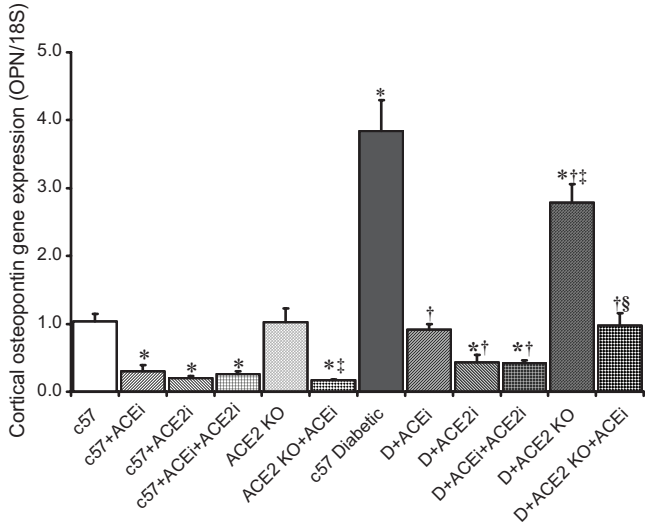

FIG. 8. The gene expression of osteopontin in cortical homogenates, as measured by real-time RT-PCR. Data are means ± SEM. $P < 0.05$ *vs. control c57, †vs. diabetic c57, ‡vs. control ACE2 KO, and §vs. diabetic ACE2 KO.

tes, consistent with its known renoprotective actions. This antifibrotic activity was preserved in diabetic *ACE2* KO mice.

The expression of osteopontin mRNA has been suggested as a useful marker of early diabetic nephropathy (22). In our study, induction of diabetes was associated with a threefold increase in cortical osteopontin gene expression in both wild-type and *ACE2* KO mice (Fig. 8). This increase was prevented by treatment with an ACE inhibitor in both wild-type and *ACE2* KO mice. Treatment with a selective ACE2 inhibitor also resulted in a reduction in osteopontin expression in both control and diabetic mice.

DISCUSSION

RAS is a key mediator of diabetic kidney disease (1). While most research has focused on the actions of ACE and Ang II, other components of the RAS may also play an important role (Fig. 1) (1). In this study, we have demonstrated that renal changes associated with early diabetes are substantially influenced by the carboxypeptidase ACE2 such that increased creatinine clearance and renal blood flow are attenuated in ACE2-deficient mice with experimental diabetes. However, diabetes-associated albuminuria was enhanced. These data point to ACE2 as a complex and site-specific modulator of diabetic kidney disease and an important new player in diabetic complications.

Increased renal blood flow in early diabetes is thought to be due to dilation of resistance arterioles, associated with an impaired reactivity to exogenous Ang II (23). However, the renal vasculature is also highly sensitive to the vasodilator effects of Ang 1–7, particularly at the afferent arteriole (24), and in states associated with activation of the local RAS, leading to an increase in effective renal plasma flow following an infusion of Ang 1–7 in rats (25). We demonstrate for the first time that the circulating ACE2 activity is significantly increased in diabetic mice, potentially contributing to impaired reactivity to Ang II (23). Moreover, suppression of this circulating angiotensinase activity with a selective ACE2 inhibitor was able to prevent diabetes-associated hyperfiltration. That similar hemodynamic patterns were observed in diabetic *ACE2* KO mice and wild-type mice receiving a selective ACE2 inhibitor suggests that circulating ACE2 may have an important role in the induction and maintenance of diabetic hyperperfusion. Although previous studies have failed to detect ACE2 activity in human plasma samples (26), the increased sensitivity of our assay and the ninefold higher ACE2 activity in murine samples compared with human plasma may partly explain this discrepancy. Moreover, the angiotensinase activity observed in this study was consistent with that of systemic ACE2, as it was inhibited by the same inhibitors (MLN-4760, EDTA, and Ang II; data not shown), and this enzyme activity was absent in *ACE2* KO mice.

A number of recent studies have shown that the expression of ACE2 is significantly altered in the diabetic kidney in a site-specific manner (6,7). For example, expression of ACE2 protein in the diabetic tubule (Fig. 2) and ACE2 mRNA levels in cortical samples is reduced in experimental diabetes (6). Consistent with this finding, we also show here that Ang 1–7, the major product of ACE2, is also significantly reduced in diabetic kidneys (Fig. 4). We and others (7) have also found that total renal ACE2 activity is paradoxically increased in the diabetic kidney (Fig. 3*B*).

We speculate that inadvertent contamination with circulating ACE2, which is elevated in diabetes, may have contributed to this finding. Although some studies have also suggested that the glomerular expression of ACE2 may be increased in experimental diabetes (6,7) and in renal biopsy samples of patients with diabetic kidney disease (27), glomeruli make up only 5% of cortical mass, suggesting that glomerular expression cannot explain differences in cortical ACE2 activity. Moreover, glomerular ACE2 expression in mice is much lower than seen in the proximal tubule (Fig. 2) or in blood.

In our study, ACE2 deficiency resulted in increased albuminuria in the context of diabetes, consistent with recent findings in the *ACE2* KO Akita mouse, which manifests increased albuminuria following the onset of diabetes (10). Similarly, Batlle and colleagues demonstrated increased albuminuria in both *db/db* mice (8) and mice with streptozotocin-induced diabetes (9) receiving the selective ACE2 inhibitor MLN-4760, injected subcutaneously at a dose of 40 mg/kg every other day. Importantly, these studies also showed that the antiproteinuric action of AT_1 receptor blockers were also partly attenuated in ACE2-deficient animals (8–10), failing to restore albuminuria to nondiabetic levels, which is normally achieved in ACE2 replete animals. In our study, we saw no reduction in albuminuria in ACE2-deficient animals treated with an ACE inhibitor. It is likely that delayed treatment with an ACE inhibitor, as employed in our study, was less renoprotective than AT_1 receptor blockers delivered from onset of diabetes in other studies, since even diabetic wild-type mice treated with an ACE inhibitor had higher levels of albuminuria than control mice. However, we wished to test the efficacy of ACE inhibition in a more clinically relevant context, reflecting the current indications for treatment in patients with type 1 diabetes with agents that block the RAS (28). Nonetheless, taken together, these findings suggest that the antiproteinuric efficacy of strategies to block the RAS in the context of diabetes is at least partly dependent on ACE2.

It is noteworthy that nondiabetic *ACE2* KO mice and mice receiving a selective ACE2 inhibitor had lower levels of albuminuria than those observed in wild-type mice, while with regard to diabetes, the opposite phenomenon was seen. It is conceivable that in nondiabetic animals, reduced renal blood flow in ACE2 deficient mice leads to reduced albuminuria. By contrast, in diabetes, the increased filtration fraction observed in diabetic ACE2-deficient mice may also have contributed to glomerular hypertension and, therein, the increased albuminuria observed in this model. In addition, we demonstrate that, in the context of diabetes, genetic or acquired ACE2 deficiency resulted in increased systolic blood pressure levels, which may also act to increase albuminuria. However, in control animals, blood pressure levels were unaffected by ACE2 deficiency. This is consistent with the lack of any hypotensive effect in healthy animals treated with Ang 1–7 itself or Ang 1–7 receptor agonists but significant blood pressure lowering with these agents in the context of diabetes (29). Ferrario and colleagues proposed that the vascular responses to Ang 1–7 (and, by association, ACE2) were augmented in conditions in which the endogenous RAS had been stimulated (4), of which diabetes is a classic example. It is possible that activation of Ang II–dependent vasoconstrictor pathways in diabetes is partly offset by upregulation of vasodilator stimuli and responsiveness (and increased renal blood flow). Furthermore, in settings where these vasodilator pathways are inhibited, such as in ACE2-deficient animals, blood pressure and albuminuria may also be increased.

In our study, the blood pressure–lowering effects of ACE inhibition were attenuated in ACE2-deficient mice both with and without diabetes. This finding is consistent with recent studies showing, firstly, that ACE inhibition significantly increases circulating Ang 1–7 levels (30) and, secondly, that preventing this increase in Ang1–7 attenuates the hypotensive effects of ACE inhibition (4). These findings contrast those made in the original description of the *ACE2* KO mouse, where the ACE inhibitor captopril reduced the blood pressure by 30–40 mmHg (11). However, in our study, we used a smaller and potentially more clinically relevant dose of the ACE inhibitor perindopril, which achieved a mean reduction in wild-type animals of only 8 mmHg. As blood pressure lowering is considered one of the major actions of ACE inhibition in the clinic, further examination of this phenomenon with radiotelemetry is warranted.

It should be noted that some of the actions of ACE inhibition, including reductions in fibronectin and osteopontin expression, were preserved in both ACE2-replete and ACE2-deficient mice with diabetes. While this finding is consistent with these renoprotective actions being fundamentally Ang II dependent, this study also showed that ACE2 inhibition was able to attenuate diabetes-associated changes in osteopontin expression and glomerular fibronectin accumulation. It is conceivable that the reduction in renal ACE activity in animals treated with a selective ACE2 inhibitor (Fig. 5) or other compensatory changes in the kidney may have indirectly contributed to the impact of ACE2 deficiency on these parameters in our study. The observed reduction in ACE expression and activity was not due to cross-reactivity of MLN-4760 with ACE, as the achieved plasma concentration of MLN-4760 when delivered orally at a dose of 10 $mg \cdot kg^{-1} \cdot day^{-1}$ ($\sim$0.03 μmol/l) is well below that for ACE ($\sim$50 μmol/l) and above that for circulating ACE2 ($\sim$5 nmol/l), which was effectively suppressed in our study (Fig. 3*C*). In addition, a reduction in cortical ACE activity was also seen in *ACE2* KO mice.

Although perindopril does not inhibit ACE2 in vitro, in our study this agent significantly reduced renal Ang 1–7 levels in nondiabetic mice. This was observed both in wild-type and *ACE2* KO mice, suggesting that this activity was not dependent on ACE2 inhibition. It has been previously shown in renal homogenates that metabolism of Ang 1–7 to peptide fragments is mediated by aminopeptidases and neutral endopeptidase (Fig. 1) rather than ACE (31). Therefore, the reduction in renal Ang 1–7 following ACE inhibition in our study probably reflects the reduction in substrate (Ang II). This contrasts with angiotensin metabolism in the circulation, wherein ACE inhibition increases Ang 1–7 levels by abolishing the generation of Ang 1–5 (30). It is possible that increased circulating levels of Ang 1–7 in wild-type animals receiving an ACE inhibitor also led to the compensatory reduction in circulating ACE2 activity observed in our study (Fig. 3*C*).

In summary, we have demonstrated that ACE2 mediates increased renal perfusion and hyperfiltration associated with diabetes. In addition, the antiproteinuric and antihypertensive actions of ACE inhibition in experimental diabetes are partly dependent on ACE2, as these effects are attenuated in diabetic *ACE2* KO mice and in animals in which circulating ACE2 activity has been pharmacologi-

cally inhibited. These data indicate that ACE2 is a complex and site-specific modulator of diabetic kidney disease and vascular dysfunction.

REFERENCES

1. Cooper ME: Pathogenesis, prevention, and treatment of diabetic nephropathy. *Lancet* 352:213–219, 1998
2. Zatz R, Dunn BR, Meyer TW, Anderson S, Rennke HG, Brenner BM: Prevention of diabetic glomerulopathy by pharmacological amelioration of glomerular capillary hypertension. *J Clin Invest* 77:1925–1930, 1986
3. Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving HH, Remuzzi G, Snapinn SM, Zhang Z, Shahinfar S: Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 345:861–869, 2001
4. Ferrario CM, Trask AJ, Jessup JA: Advances in biochemical and functional roles of angiotensin-converting enzyme 2 and angiotensin-(1–7) in regulation of cardiovascular function. *Am J Physiol Heart Circ Physiol* 289: H2281–H2290, 2005
5. Li N, Zimpelmann J, Cheng K, Wilkins JA, Burns KD: The role of angiotensin converting enzyme 2 in the generation of angiotensin 1–7 by rat proximal tubules. *Am J Physiol Renal Physiol* 288:F353–F362, 2005
6. Tikellis C, Johnston CI, Forbes JM, Burns WC, Burrell LM, Risvanis J, Cooper ME: Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. *Hypertension* 41:392–397, 2003
7. Wysocki J, Ye M, Soler MJ, Gurley SB, Xiao HD, Bernstein KE, Coffman TM, Chen S, Batlle D: ACE and ACE2 activity in diabetic mice. *Diabetes* 55:2132–2139, 2006
8. Ye M, Wysocki J, William J, Soler MJ, Cokic I, Batlle D: Glomerular localization and expression of angiotensin-converting enzyme 2 and angiotensin-converting enzyme: implications for albuminuria in diabetes. *J Am Soc Nephrol* 17:3067–3075, 2006
9. 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* 72:614–623, 2007
10. 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* 171:438–451, 2007
11. Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-dos-Santos AJ, da Costa J, Zhang L, Pei Y, Scholey J, Ferrario CM, Manoukian AS, Chappell MC, Backx PH, Yagil Y, Penninger JM: Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* 417:822–828, 2002
12. 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* 168:1808–1820, 2006
13. Allen TJ, Cooper ME, Lan HY: Use of genetic mouse models in the study of diabetic nephropathy. *Curr Diab Rep* 4:435–440, 2004
14. Buñag R: Validation in awake rat of a tail-cuff method for measurement of systolic blood pressure. *J Appl Physiol* 34:279–282, 1973
15. Cefalu WT, Wang ZQ, Bell-Farrow A, Kiger FD, Izlar C: Glycohemoglobin measured by automated affinity HPLC correlates with both short-term and long-term antecedent glycemia. *Clin Chem* 40:1317–1321, 1994
16. Dunn SR, Qi Z, Bottinger EP, Breyer MD, Sharma K: Utility of endogenous creatinine clearance as a measure of renal function in mice. *Kidney Int* 65:1959–1967, 2004
17. Thomas MC, Jerums G, Tsalamandris C, Macisaac R, Panagiotopoulos S, Cooper ME: Increased tubular organic ion clearance following chronic ACE inhibition in patients with type 1 diabetes. *Kidney Int* 67:2494–2499, 2005
18. Thomas MC, Tikellis C, Burns WM, Bialkowski K, Cao Z, Coughlan MT, Jandeleit-Dahm K, Cooper ME, Forbes JM: Interactions between renin angiotensin system and advanced glycation in the kidney. *J Am Soc Nephrol* 16:2976–2984, 2005
19. Forbes JM, Thallas V, Thomas MC, Founds HW, Burns WC, Jerums G, Cooper ME: The breakdown of preexisting advanced glycation end products is associated with reduced renal fibrosis in experimental diabetes. *FASEB J* 17:1762–1764, 2003
20. Zimpelmann J, Kumar D, Levine DZ, Wehbi G, Imig JD, Navar LG, Burns KD: Early diabetes mellitus stimulates proximal tubule renin mRNA expression in the rat. *Kidney Int* 58:2320–2330, 2000
21. Burns KD: Angiotensin II and its receptors in the diabetic kidney. *Am J Kidney Dis* 36:449–467, 2000
22. Susztak K, Bottinger E, Novetsky A, Liang D, Zhu Y, Ciccone E, Wu D, Dunn S, McCue P, Sharma K: Molecular profiling of diabetic mouse kidney reveals novel genes linked to glomerular disease. *Diabetes* 53:784–794, 2004
23. Inman SR, Porter JP, Fleming JT: Reduced renal microvascular reactivity to angiotensin II in diabetic rats. *Microcirculation* 1:137–145, 1994
24. Ren Y, Garvin JL, Carretero OA: Vasodilator action of angiotensin-(1–7) on isolated rabbit afferent arterioles. *Hypertension* 39:799–802, 2002
25. Sampaio WO, Nascimento AA, Santos RA: Systemic and regional hemodynamic effects of angiotensin-(1–7) in rats. *Am J Physiol Heart Circ Physiol* 284:H1985–H1994, 2003
26. Elased KM, Cunha TS, Gurley SB, Coffman TM, Morris M: New mass spectrometric assay for angiotensin-converting enzyme 2 activity. *Hypertension* 47:1010–1017, 2006
27. Lely AT, Hamming I, van Goor H, Navis GJ: Renal ACE2 expression in human kidney disease. *J Pathol* 204:587–593, 2004
28. American Diabetes Association: Standards of medical care in diabetes–2007. *Diabetes Care* 30 Suppl. 1:S4–S41, 2007
29. Benter IF, Yousif MH, Cojocel C, Al-Maghrebi M, Diz DI: Angiotensin-(1–7) prevents diabetes-induced cardiovascular dysfunction. *Am J Physiol Heart Circ Physiol* 292:H666–H672, 2007
30. Yamada K, Iyer SN, Chappell MC, Ganten D, Ferrario CM: Converting enzyme determines plasma clearance of angiotensin-(1–7). *Hypertension* 32:496–502, 1998
31. Allred AJ, Diz DI, Ferrario CM, Chappell MC: Pathways for angiotensin-(1–7) metabolism in pulmonary and renal tissues. *Am J Physiol Renal Physiol* 279:F841–F850, 2000