Original Article

ACE and ACE2 Activity in Diabetic Mice

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ACE-related carboxypeptidase (ACE2) may counterbalance the angiotensin (ANG) II–promoting effects of ACE in tissues where both enzymes are found. Alterations in renal ACE and ACE2 expression have been described in experimental models of diabetes, but ACE2 activity was not assessed in previous studies. We developed a microplate-based fluorometric method for the concurrent determination of ACE and ACE2 activity in tissue samples. Enzymatic activity (relative fluorescence unit [RFU] · μg protein^{-1} · h^{-1}) was examined in ACE and ACE2 knockout mice and in two rodent models of diabetes, the *db/db* and streptozotocin (STZ)-induced diabetic mice. In kidney cortex, preparations consisting mainly of proximal tubules and cortical collecting tubules, ACE2 activity had a strong positive correlation with ACE2 protein expression (90-kDa band) in both knockout models and their respective wild-type littermates ($r = 0.94$, $P < 0.01$). ACE activity, likewise, had a strong positive correlation with renal cortex ACE protein expression (170-kDa band) ($r = 0.838$, $P < 0.005$). In renal cortex, ACE2 activity was increased in both models of diabetes (46.7 ± 4.4 vs. 22.0 ± 4.7 in *db/db* and *db/m*, respectively, $P < 0.01$, and 22.1 ± 2.8 vs. 13.1 ± 1.5 in STZ-induced diabetic versus untreated mice, respectively, $P < 0.05$). ACE2 mRNA levels in renal cortex from *db/db* and STZ-induced diabetic mice, by contrast, were not significantly different from their respective controls. In cardiac tissue, ACE2 activity was lower than in renal cortex, and there were no significant differences between diabetic and control mice (*db/db* 2.03 ± 0.23 vs. *db/m* 1.85 ± 0.10; STZ-induced diabetic 0.42 ± 0.04 vs. untreated 0.52 ± 0.07 mice). ACE2 activity in renal cortex correlated positively with ACE2 protein in *db/db* and *db/m* mice ($r = 0.666$, $P < 0.005$) as well as in STZ-induced diabetic and control mice ($r = 0.621$, $P < 0.05$) but not with ACE2 mRNA ($r = -0.468$ and $r = -0.522$, respectively). We conclude that in renal cortex from diabetic mice, ACE2 expression is increased at the posttranscriptional level. The availability of an assay for concurrent measurement of ACE and ACE2 activity should be helpful in the evaluation of kidney-specific alterations in the balance of these two carboxypeptidases, which are involved in the control of local ANG II formation and degradation. *Diabetes* 55:2132–2139, 2006

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ACE is a monomeric, membrane-bound, zinc- and chloride-dependent peptidyl dipeptidase that catalyzes the conversion of angiotensin (ANG) I to ANG II by removing a carboxy-terminal dipeptide (1). More recently, an ACE-related carboxypeptidase (ACE2) has been identified in humans and differs from ACE in that it preferentially removes carboxy-terminal hydrophobic or basic amino acids (2,3). ACE2 is the only known enzymatically active homologue of ACE in the human genome (1). ANG I and II, as well as other biologically active peptides, are substrates for ACE2 (1). ACE2 activity may counterbalance the ANG II promoting effects of ACE by preventing ANG II accumulation in tissues where ACE2 and ACE are both expressed (4–6).

The renin-ANG system is the target of therapies to slow disease progression in diabetic and nondiabetic kidney disease (7–10). Alterations in ACE (11) and both ACE and ACE2 have been described in models of diabetic kidney disease (5,6). There is also increasing interest in ACE2 as a regulator of the fate of ANG peptides in hypertension and vascular and pulmonary pathology (4,12–14). In view of the recent interest in ACE2 in diabetes and other pathological conditions, it would be important to have assays that measure tissue activity accurately and conveniently. Moreover, a method for concomitant measurement of ACE and ACE2 activity would be helpful to evaluate the relative effects of these two carboxypeptidases at the tissue level in various pathophysiological states. Recently, Huang et al. (15) reported that 7-methoxycoumarin-Tyr-Val-Ala-Asp-Ala-Pro-Lys (2,4-dinitrophenyl) (7-Mca-YVADAPK[Dnp]) is a fluorogenic substrate that can be cleaved by both ACE and ACE2. However, no data exist on the use of this substrate for measurement of ACE or ACE2 in body fluids or tissues. In this study, we describe a new and easy-to-use method for simultaneous measurements of tissue ACE and ACE2 activity. We utilized this assay to measure the activity of these enzymes in kidney and cardiac tissue from two animal models of diabetes. Moreover, the enzymatic activity was examined in conjunction with the levels of protein and mRNA expression.

RESEARCH DESIGN AND METHODS

ACE knockout mice (ACE.4 mice) have the somatic ACE promoter replaced by the kidney androgen-regulated protein promoter (16). This modification was intended to produce androgen-responsive expression of ACE in the kidney. However, the kidney androgen-regulated protein promoter is nonfunctional in these mice, and ACE is not detectable in organs other than the kidney (16). The levels of renal ACE are only ~1–2% of normal. Blood pressure and plasma ANG II levels of the ACE.4 knockout mice are significantly lower than those of wild-type animals (16,17).

ACE2-deficient mice are viable, are capable of efficient reproduction, and

lack any gross anatomical or structural abnormalities (18). They do not appear to have significant cardiac dysfunction. Daytime baseline systolic blood pressure and baseline levels of plasma ANG II are similar in *ACE2*-deficient and wild-type control mice (18). Following acute ANG II infusion, however, plasma concentrations of ANG II increase to levels that are almost threefold higher in *ACE2*-deficient mice than controls (18). Chronic infusion of ANG II, at levels that do not significantly alter blood pressure in wild-type controls, increases systolic blood pressure by 32 mmHg in *ACE2*-deficient mice. This increase in blood pressure is associated with enhanced accumulation of ANG II in the kidney (18)

Eight-week-old female *db/db* mice (C57BLKS/J^{Lepr}) were used as a model of type 2 diabetes, and their lean littermates (*db/m*) served as nondiabetic controls (The Jackson Laboratories, Bar Harbor, ME). At the age at which the animals were killed, *db/db* mice had markedly elevated levels of blood glucose (*db/db* 19.8 ± 2.1 vs. *db/m* 9.2 ± 0.5 mmol/l, $P < 0.001$) as measured with a One Touch Ultra glucometer (LifeScan, Mountain View, CA). The animals at this age have modest albuminuria, but light microscopy is essentially normal (19).

As a model of type 1 diabetes, streptozotocin (STZ)-induced diabetic mice were used. Diabetes was induced in female C57BL/6J mice by two intraperitoneal injections of STZ (Sigma Aldrich, St. Louis, MO) at a dose of 150 μg/g body wt in 50 μl of sterile 0.05 mol/l sodium citrate (pH 4.5). The control group received an equal volume of vehicle. Mice did not receive insulin and were killed 7 weeks after STZ injections. All STZ-induced diabetic mice developed stable hyperglycemia (STZ 24.3 ± 2.7 vs. controls 8.6 ± 0.4 mmol/l, $P < 0.005$). At this early time point, the major histologic feature is glomerular hypertrophy, so it is likely that hemodynamic changes contribute to the development of albuminuria (20). The institutional animal care and use committee approved all procedures.

RNA isolation and RT/real-time PCR. RNA was isolated from renal cortex with Trizol reagent (GIBCO Invitrogen). Quantitative real-time PCR of kidney tissue samples was performed using the TaqMan Gold RT-PCR kit and ABI Prism 7700 (Applied Biosystems) sequence-detection system. Primers and probes for ACE and ACE2 were designed using Primer Express software (Applied Biosystems). The forward primer, reverse primer, and probe were as follows: for *ACE2* gene (GenBank no. BC026801) 5′-GGATACCTACCCTTC CTACATCAGC-3′, 5′-CTACCCCACATATCACCAAGCA-3′ and 5′-FAM-CCA CTGGATGCCTCCCTGCCC-TAMRA-3′ and for *ACE* gene (GenBank no. BC040404): 5′CAGAATCTACTCCACT GGCAAGGT-3′, 5′-TCGTGAGGAAG CCAGGATGT-3′, and 5′-FAM-CAACAAGACTGC CACCTGCTGGTCC-TAMRA-3′. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control (GenBank no. XM354601), which included forward primer, 5′ CAGAAGACT GTGGATGGCCCCTC-3′; reverse primer, 5′-TGCACCACCAACTGCTTAG-3′; and probe, 5′-FAM CAGAAGACTGTGGATGGCCCCTC-TAMRA-3′. Reverse transcription was carried out for 30 min at 48°C. The samples were heated to 95°C for 10 min, and 40 cycles of a two-step PCR were performed (95°C for 15 s and 60°C for 60 s). The ACE and ACE2 mRNA levels of the samples were normalized to their glyceraldehyde-3-phosphate dehydrogenase contents. Experiments were carried out in triplicate for each data point.

Membrane protein preparation and Western blot analysis. Membrane proteins from kidney tissue were isolated as previously described (5). Protein concentrations were determined by the bicinchoninic acid method (Pierce). Membrane protein lysates (50 μg) were loaded into SDS-PAGE (10% gels), and ACE and ACE2 proteins were detected by Western blot, as previously specified (5). Monoclonal antibody against ACE (2E2; Chemicon) diluted 1/200 and noncommercial affinity-purified anti-ACE2 antibody (12) were used to detect ACE and ACE2 protein, respectively. To ensure equal loading of proteins, membranes were stripped and then reprobed with an antibody against β-actin (Sigma). Signals on Western blots were quantified by densitometry and corrected for the β-actin signal, using an Eagle Eye II Still Video System.

ACE2 and ACE enzyme activity assay. The ability to cleave the fluorogenic substrate, 7-Mca-YVADAPK(Dnp) (R&D Systems), by purified ACE and ACE2 protein was used to assess the respective activity of ACE and ACE2 concurrently in tissue samples. Cleavage of this substrate by either enzyme removes the 2,4-dinitrophenyl moiety that quenches the fluorescence of the 7-methoxycoumarin moiety, thus resulting in increased fluorescence (15,21). To prevent undesirable hydrolysis of the substrate by a range of nonmetalloprotease enzymes from mouse tissues, all tests were performed with the addition of an inhibitor cocktail (complete EDTA-free tablets; Roche).

The ACE inhibitor captopril and a carboxypeptidase A inhibitor, benzyl succinate (Sigma), failed to quench fluorescence when incubated with human recombinant (hr) ACE2 (20 nmol/l, R&D systems) at a concentration up to 100 μmol/l (data not shown). This is in agreement with previous studies (2,15). We tested the effect of two different ACE2 inhibitors, MLN-4760 (Millennium Pharmaceuticals) and DX600 (Phoenix Pharmaceuticals), at concentrations ranging from 100 pmol/l to 100 μmol/l on hrACE2. MLN-4760 quenched the signal completely at 1–10 nmol/l, whereas a higher concentration (100 nmol/l) of DX600 was needed to achieve complete quenching of the signal. A higher ACE2 inhibitory potency of MLN-4760 over DX600 has been reported previously (15,22). Therefore, further studies were done using MLN-4760. In tissue extracts, the concentrations of MLN-4760 required for fluorescence quenching were high and more variable. Near-maximal inhibition of the fluorescence signal, calculated per microgram total protein, was achieved at a concentration of MLN-4760 ranging from 10 μmol/l to 1 mmol/l. Tissue samples (kidney cortex and heart) were homogenized in a buffer consisting of (in mmol/l) 50 HEPES, pH 7.4, 150 NaCl, 0.5% Triton X-100, 0.025 $ZnCl_2$, and 1.0 phenylthanolamine-*N*-methyltransferase and then clarified by centrifugation at 10,000*g* for 15 min. After measuring protein concentration, tissue samples were diluted in a buffer (50 mmol/l 4-morpholineethanesulfonic acid, 300 mmol/l NaCl, 10 μmol/l $ZnCl_2$, and 0.01% Triton-X-100, pH 6.5), containing EDTA-free tablets. To each well, 88 μl of a diluted tissue sample (1 μg of total protein/well for kidney tissue extracts and 10 μg/well for heart tissue) was added, along with 10 μl of buffer (with the respective inhibitor), and the reaction was initiated by the addition of 2 μl of the substrate (1.0 μmol/l, final concentration). The plates were read using a fluorescence plate reader FLX800 (BIOTEK Instruments) at an excitation wavelength of 320 nm and an emission wavelength of 400 nm. All reactions were performed at ambient temperature in microtiter plates with a 100 μl total volume.

Kidney cortex samples were incubated at room temperature to assess the time dependency of the fluorescence signal. As with other fluorophores, the signal resulting from 7-Mca-YVADAPK(Dnp) hydrolysis increased with time (23). Fluorescence readings in both the absence and presence of MLN-4760 (1 mmol/l) and captopril (10 umol/l) were near maximal after 4 h of monitoring, and therefore this time-point was chosen for the studies. We recommend that the assay be run at this time point because after 4 h, subsequent digestion of the products of ACE and ACE2 activity occurs and therefore may interfere with the enzyme activity measurements in tissue samples.

Background fluorescence readings over time were obtained from reactions without tissue samples. No substantial increase in fluorescence was noted even after 24 h incubation, indicating that there is no significant spontaneous substrate hydrolysis under the reaction conditions.

In a set of experiments, kidney tissue samples lacking ACE2 or ACE obtained from the respective knockout mice were spiked with increasing amounts of exogenous human recombinant ACE2 (R&D Systems) or human ACE standard (ACE Kinetic; Bűhlmann Laboratories, Allschwil, Switzerland), and the resultant increase in fluorescence was recorded.

Defining tissue ACE and ACE2 activity. We used a fluorogenic substrate, 7-Mca-YVADAPK(Dnp), which is cleaved by purified ACE and ACE2 metalloproteases. An effect of another metalloprotease, carboxypeptidase A, on this substrate was ruled out by showing that a specific inhibitor, benzyl succinate (100 umol/l) (3), did not reduce significantly the fluorescence signal in kidney cortex (Fig. 1). Similar results were obtained for heart tissue (not shown). This indicates that carboxypeptidase A does not interfere with measurement of ACE and ACE2 activity using this substrate in kidney and heart tissue. EDTA (1 mmol/l) quenched the fluorescence down to ~16% of control (Fig. 1). EDTA chelates the zinc ion required for metalloprotease activity (24). ACE inhibition, using captopril, decreased the signal to 24.9 ± 1.1% of the control, whereas specific ACE2 inhibitor, MLN-4760, reduced fluorescence intensity significantly to 46.4 ± 0.7% of the control (Fig. 1). The concomitant use of captopril and MLN-4760 nearly completely quenched the fluorescence signal (7.7 ± 0.9% of control) (Fig. 1).

To account for the effect of ACE on the fluorogenic substrate while measuring ACE2 activity, the ACE2-dependent signal was measured in the presence of captopril. Conversely, when measuring ACE activity, tissue samples were incubated with MLN-4760.

ACE and ACE2 activity were defined as follows: ACE activity = A−C, where A is fluorescence in the presence of the ACE2 inhibitor (MLN-4760) and reflects the ACE2 inhibitor-resistant signal and C is fluorescence in the presence of both captopril and MLN-4760 and is a reflection of both ACE2 and ACE inhibitor-resistant signals combined. ACE2 activity = B−C, where B is the fluorescence in the presence of the ACE inhibitor, captopril, and thereby reflects ACE inhibitor-resistant signal; C again is the fluorescence, which is resistant to both ACE and ACE2 inhibitor.

The results reported therein are all based on these formulas. We want to note, however, that ACE activity could also be defined as the difference between fluorescence without inhibitors and fluorescence remaining after inhibition with the ACE inhibitor, captopril. Likewise, ACE2 activity could be defined as the difference between fluorescence without inhibitors and fluorescence remaining after inhibition with the ACE2 inhibitor, MLN-4760. In pilot studies, we found strong positive correlations for both ACE ($r = 0.754$, $n = 9$) and ACE2 activity ($r = 0.964$, $n = 9$) calculated with each of the above two formulas for ACE and ACE2. Moreover, in kidney cortex spiked with exogenous ACE and ACE2, both formulas gave similar data in terms of recovery of the respective ACE and ACE2 activity.

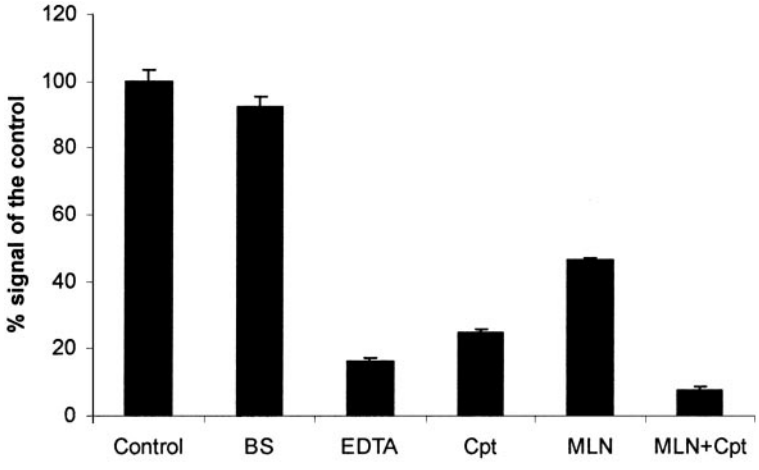

FIG. 1. Fluorescence under control conditions (100%) and after addition of different enzyme inhibitors: 100 μmol/l benzyl succinate (BS), 1 mmol/l EDTA, ACE inhibitor Captopril (Cpt; 10 μmol/l), and the specific ACE2 inhibitor MLN-4760 (MLN; 1 mmol/l). The columns represent means ± SE calculated from triplicate wells and are expressed as percent of to the values obtained in control experiments.

Intra- and interassay variability and comparison with other methods. The method for measuring enzymatic activity for ACE2 and ACE had intra- and interassay coefficients of 14.7 and 10.1%, respectively. We compared our method to a widely used colorimetric method for measurement of tissue ACE activity (ACE color; Fujirebio) (5,25–27). A strong correlation was found between the two methods for ACE activity using renal cortex ($r = 0.980$, $P < 0.001$, $n = 13$). We could not compare our method for ACE2 activity to the one used by Burrell et al. (28) in rat hearts because in mouse tissues, cleavage of the substrate they used ([7-methoxycoumarin-4-yl]-acetyl-Ala-Pro-Lys [2,4-dinitrophenyl] [custom synthesized by Genemed Synthesis, South San Francisco, CA]) was not quenchable by ACE2 inhibitor (MLN-4760, 1 mmol/l) (data not shown). We also assessed whether our approach to measure ACE2 activity could be applied to mouse serum. As anticipated from previous work with ACE2 (29), no enzymatic activity was detectable in serum by our assay. Enzymatic activity measurements in mouse kidney cortex using two different concentrations of MLN-4760 (10 μmol/l and 1 mmol/l) showed a good correlation for both ACE ($r = 0.991$, $n = 6$) and ACE2 activity ($r = 0.858$, $n = 12$).

Statistical analysis. Results are expressed as means ± SE. Statistical comparisons between groups were performed by Mann-Whitney test. Correlations between two variables were determined by linear regression analysis. Statistical significance was accepted at a value of $P < 0.05$.

RESULTS

Renal ACE2 and ACE activity in kidneys from ACE and ACE2 knockout. ACE2 and ACE activity were examined in models of ACE2 and ACE gene ablation (ACE2 and ACE knockout mice) and the respective wild types as a way to investigate enzymatic activity over a wide range of ACE2 and ACE protein expression. In kidney samples from ACE knockout mice, the band corresponding to ACE protein was not detectable by Western blot (Fig. 2*A*). ACE activity was markedly reduced in ACE-deficient mice (ranging from 4.6 to 6.2% of the activity values measured in wild-type kidneys). In the ACE2 wild type, Western blot of kidney cortex with a specific ACE2 antibody revealed bands at ~67 and 90 kDa (Fig. 2*B*). The presence of these two bands was abolished with the respective blocking peptide used for raising the antibody. In the ACE2 knockout, by contrast, the 90-kDa band was absent (Fig. 2*B*). ACE2 activity in kidneys from ACE2 knockout was barely detectable, ranging from −3.2 to 7.9% of the activity values measured in wild-type mice.

When we plotted ACE2 activity in kidney tissues from two knockouts and their respective wild types over the range of ACE2 protein expression, a strong positive correlation was found between ACE2 protein abundance and activity in kidney cortex ($r = 0.94$, $P < 0.01$, $n = 10$). By contrast, renal cortex ACE2 activity had no correlation with renal cortex ACE protein expression ($r = -0.02$, $P =$ NS), documenting further the specificity of our assay for ACE2.

ACE activity also had a strong positive correlation with renal cortex ACE protein expression (the 170-kDa band recognized by monoclonal ACE antibody) in both knockout models and their respective wild-type littermates ($r = 0.838$, $n = 10$, $P < 0.005$).

To further examine ACE activity as a function of ACE protein, we added human ACE standard to kidney extract obtained from ACE knockout mice. This resulted in an increase in fluorescence signal in a dose-dependent manner with a linear relationship ($r = 0.988$, $P < 0.001$) ranging from 0.0625 to 1.0 mIU/well (Fig. 3*A*). To examine ACE2 activity as a function of ACE2 protein in kidney tissue, we added purified human recombinant (hr)ACE2 protein to kidney tissue extract from ACE2 knockout mouse. In these "spiking" experiments, the fluorescence

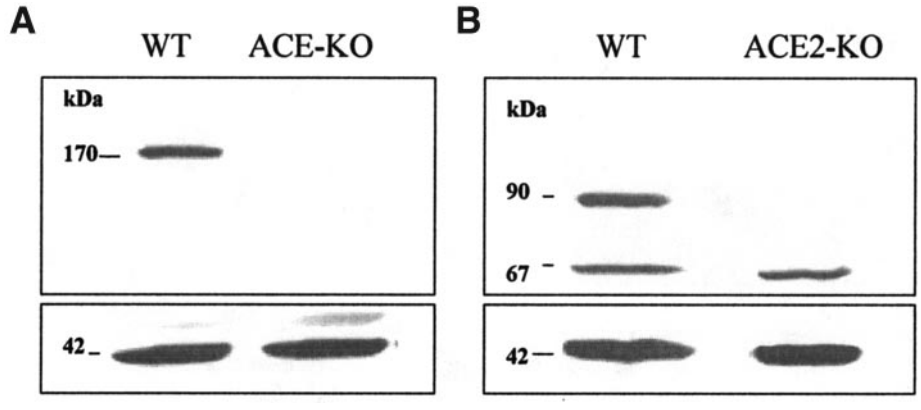

FIG. 2. ACE and ACE2 protein in renal cortex of ACE and ACE2 knockout (KO) and their respective wild-type (WT) littermates. After probing with antibody against ACE (*A*, *top*) or ACE2 (*B*, *top*), nitrocellulose was reprobed for β-actin (band at 42 kDa; *A* and *B*, *bottom*) to account for loading differences. *A*: A representative Western blot of membrane protein preparations demonstrating the presence of 170 kDa band detected by ACE antibody in WT, which was undetectable in ACE-KO mouse. *B*: ACE2 antibody detected 90 kDa protein in WT but not in ACE2-KO mouse. An ACE2 immunoreactive protein at ~67 kDa was detected in both WT and ACE2-KO mice.

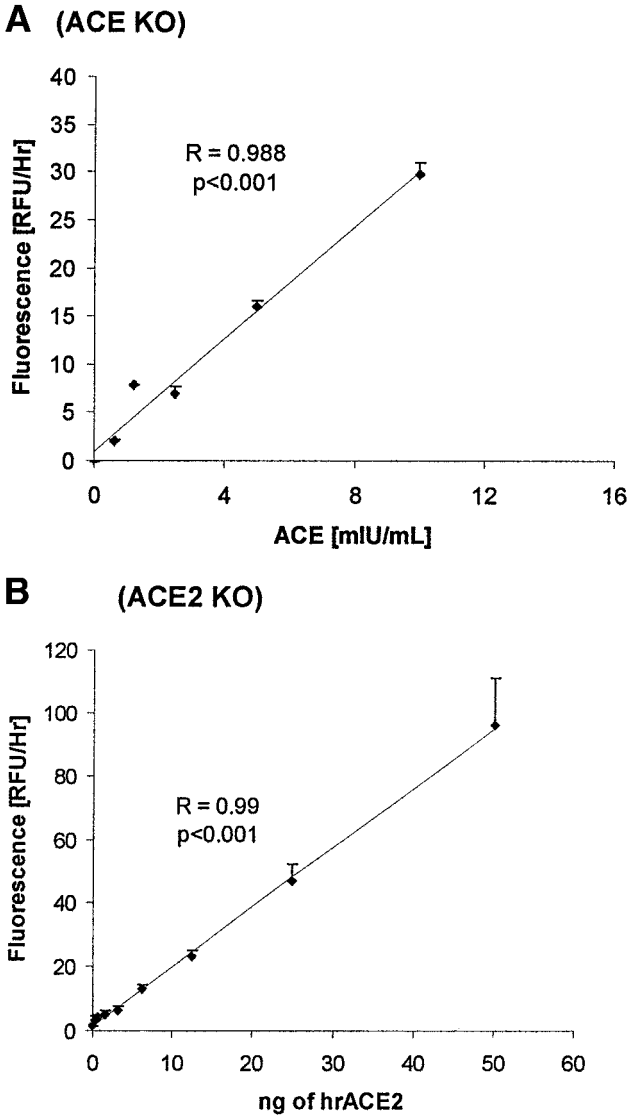

FIG. 3. Spiking experiments in kidney cortex samples from ACE and ACE2 knockout mice. Recovery of ACE (*A*) and ACE2 (*B*) activity expressed as fluorescence (RFU/h) after addition of either human ACE standard or human recombinant ACE2, respectively, is linear and highly correlated to the amount of the spiked protein.

signal was recovered in a dose-dependent manner with a linear relationship ($r = 0.990$, $P < 0.001$) in the range of 0.4–50 ng ACE2 protein/well (Fig. 3*B*). The average activity in tissue extracts from wild-type mice corresponded to a concentration of 15 ng hACE2/μg total protein by comparison with purified recombinant enzyme under identical conditions. As little as 0.4 ng of hACE2 was detectable in spiked renal cortex tissue from the ACE2 knockout mouse, suggesting an excellent detection limit of ACE2 activity of our assay.

ACE/ACE2 activity in kidney cortex from diabetic mice. ACE2 activity was measured in two different mouse models of diabetes: the *db/db* and STZ-induced diabetic mice and their nondiabetic littermates. In kidney cortex, ACE2 activity was increased in both models of diabetes as compared with their respective controls (*db/db* 46.7 ± 4.4 vs. *db/m* 22.0 ± 4.7, $P < 0.01$; STZ 22.1 ± 2.8 vs. non-STZ controls 13.1 ± 1.5 RFU · μg protein^{-1} · h^{-1}, $P < 0.05$, respectively). The differences in enzymatic activity between the controls used for both diabetic models likely reflect their different genetic background (C57BLKS vs. C57BL/6J).

ACE2 protein levels were significantly increased in renal cortex from the *db/db* and STZ-induced diabetic mice as compared with those from their respective controls (Fig. 4*A* and *B*). In contrast to ACE2 protein and activity, however, ACE2 mRNA levels were not significantly different between renal cortex from control and diabetic mice (Fig. 4*A* and *B*). This is in sharp contrast to ACE expression, which was altered not only at the protein and activity level but at the mRNA level as well in both *db/db* and STZ-induced diabetic mice (Fig. 4*C* and *D*).

We also examined ACE and ACE2 activity in relation to renal cortical ACE and ACE2 gene and protein expression. When the data from *db/m* and *db/db* mice were pooled, ACE activity showed a significant positive correlation with both ACE mRNA ($r = 0.744$, $P < 0.05$, $n = 8$) (Fig. 5*A*) and ACE protein levels ($r = 0.711$, $P < 0.01$, $n = 17$) (Fig. 5*B*). ACE2 activity also correlated positively with ACE2 protein in kidney tissue from *db/db* and *db/m* mice pooled together ($r = 0.666$, $P < 0.005$, $n = 17$) (Fig. 5*D*). ACE2 activity, however, did not correlate positively with mRNA levels measured by real-time PCR. In fact, a negative correlation was found that did not reach statistical significance ($r = -0.468$, NS, $n = 16$) (Fig. 5*C*). ACE2 mRNA levels also did not correlate significantly with protein levels ($r = 0.119$, NS, $n = 16$).

ACE activity in renal cortex from STZ-induced diabetic mice and their respective controls showed significant positive correlation with both ACE mRNA ($r = 0.681$, $P < 0.05$, $n = 15$) (Fig. 6*A*) and protein levels ($r = 0.692$, $P < 0.05$, $n = 15$) (Fig. 6*B*). ACE2 activity also correlated positively with ACE2 protein in kidney tissue from STZ-induced diabetic mice and non-STZ controls ($r = 0.621$, $P < 0.05$, $n = 15$) (Fig. 6*D*). There was, however, no positive correlation between ACE2 activity and mRNA levels. In fact, a negative correlation was found, and this reached statistical significance ($r = -0.522$, $P < 0.05$, $n = 15$) (Fig. 6*C*). ACE2 mRNA levels also did not correlate significantly with ACE2 protein levels ($r = -0.395$, NS, $n = 15$). Taken together, these findings suggest that ACE2, unlike ACE, is altered at the posttranscriptional level in kidneys from both models of diabetes.

To examine whether the increase in ACE2 activity in diabetic mice correlates with hyperglycemia, ACE2 activity was plotted against blood glucose levels. ACE2 activity showed a strong positive correlation with blood glucose levels in STZ-induced diabetic mice and their nondiabetic controls pooled together (Fig. 7*A*). Moreover, a significant positive correlation between ACE2 activity and blood glucose levels was found in kidneys from *db/m* and *db/db* mice pooled together (Fig. 7*B*). A positive correlation was also found between blood glucose levels and ACE2 protein in STZ-induced diabetic and untreated mice ($r = 0.647$, $P < 0.01$, $n = 15$) as well as *db/db* and *db/m* mice ($r = 0.610$, $P < 0.05$, $n = 15$) but not between blood glucose and ACE2 mRNA levels ($r = -0.737$, $P < 0.005$, $n = 15$; and $r = -0.137$, NS, $n = 15$, respectively).

ACE/ACE2 activity in heart from diabetic mice. Cardiac ACE2 activity was not significantly different between diabetic and control mice (*db/db* 2.03 ± 0.23 vs. *db/m* 1.85 ± 0.10; and STZ 0.42 ± 0.04 vs. controls 0.52 ± 0.07 RFU · μg protein^{-1} · h^{-1}). ACE activity measured in the hearts of diabetic mice also did not differ significantly from the respective nondiabetic controls (*db/db* 2.03 ± 0.37 vs.

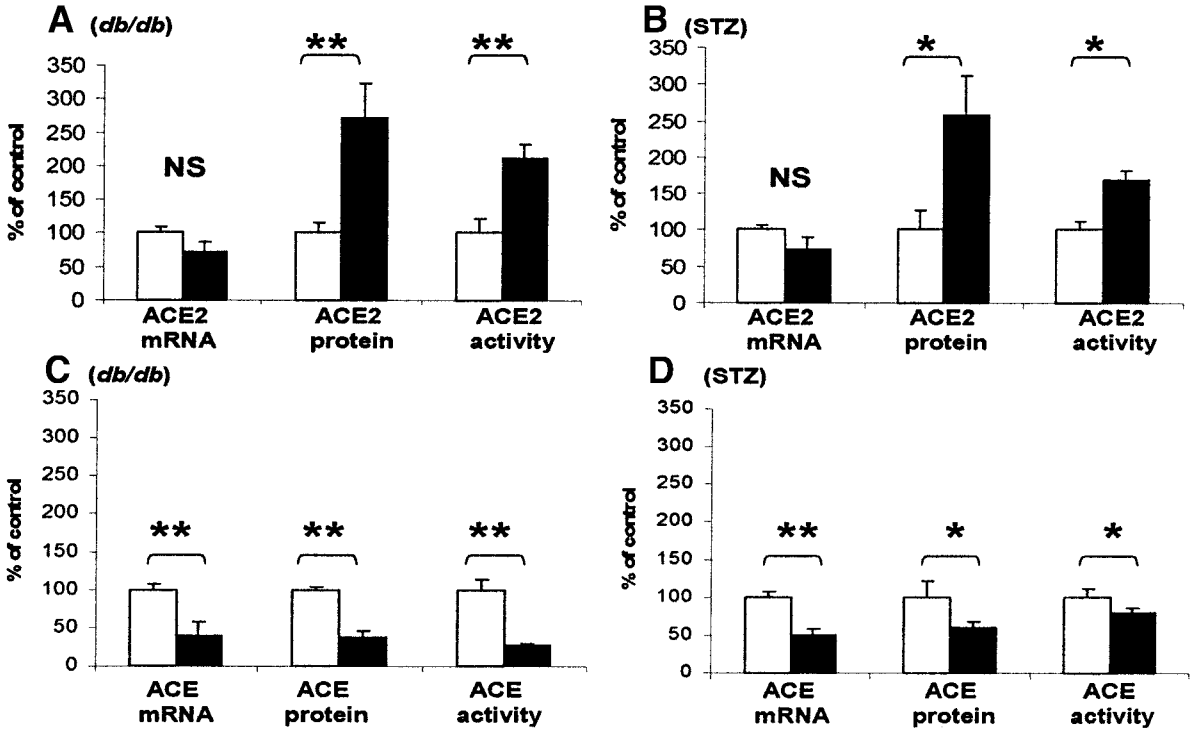

FIG. 4. ACE2 (*A* and *B*) and ACE (*C* and *D*) mRNA protein and enzymatic activity levels in kidney cortex from diabetic *db/db* (*A* and *C*) and STZ-induced diabetic mice (*B* and *D*) expressed as percent of the values measured in their respective nondiabetic controls. ■, diabetic mice; □, controls. **P* < 0.05; ***P* < 0.01.

db/m 2.53 ± 0.30 and STZ 0.630 ± 0.10 vs. non-STZ 0.628 ± 0.07 RFU · μg protein^{-1} · h^{-1}).

The level of ACE2 activity per ug total protein was ~10- to 30-fold higher in kidney cortex than in the heart (see respective values, above). The level of ACE activity was also several fold higher in kidney cortex than in cardiac tissue in both control and diabetic mice. These differences in the level of enzymatic activity between kidney and heart tissue likely reflect that ACE and ACE2 are both abundantly expressed in renal proximal tubules, which repre-

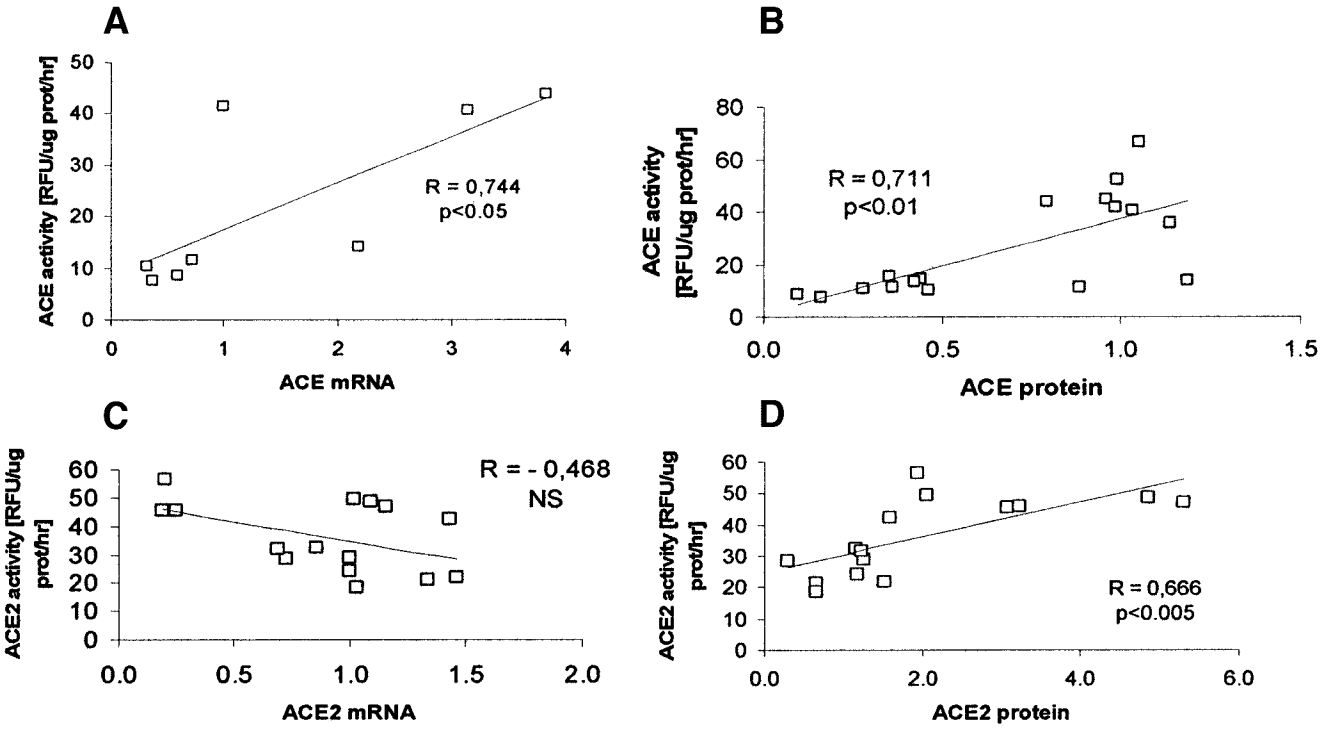

FIG. 5. Correlation between mRNA and enzymatic activity (*A* and *C*) and between protein and enzymatic activity (*B* and *D*) for ACE (*A* and *B*) and ACE2 (*C* and *D*) in renal cortex tissue extracts from *db/m* and *db/db* mice.

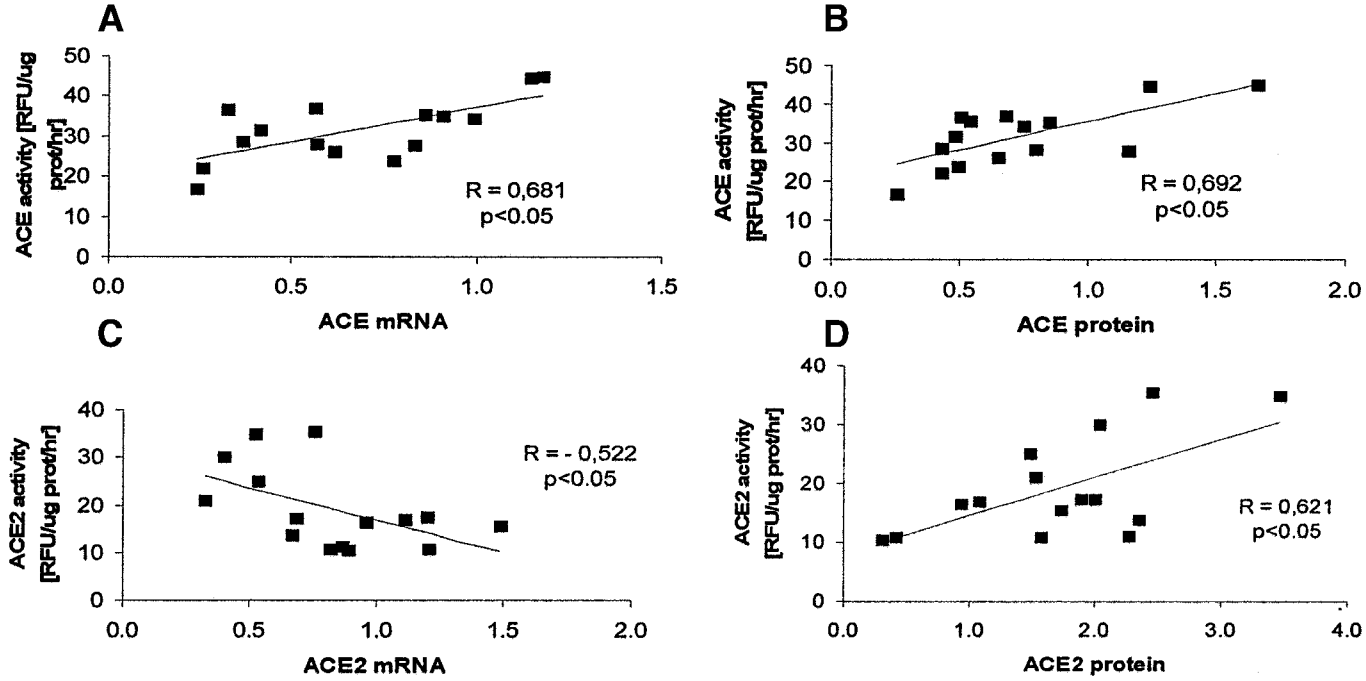

FIG. 6. Correlation between mRNA and enzymatic activity (*A* and *C*) and between protein and enzymatic activity (*B* and *D*) for ACE (*A* and *B*) and ACE2 (*C* and *D*) in renal cortex tissue extracts from STZ-induced diabetic mice and their respective controls.

sent much of the kidney cortex preparation used in these studies.

DISCUSSION

Alterations in ACE and ACE2 have been described in two different rodent models of diabetes, the STZ-induced diabetic rats (6,11) and the *db/db* mice (5), but ACE2 activity was not measured in these previous studies. In this study, we found that renal ACE2 activity was markedly increased in *db/db* and STZ-treated mice (Fig. 4*A* and *B*). This increase was associated with an increase of ACE2 protein but not ACE2 mRNA. ACE activity, by contrast, was associated with reduced mRNA levels as well. Thus, in diabetes, ACE2 is altered at the posttranscriptional level. We found a highly significant correlation between ACE2 protein abundance and enzymatic activity when data from two models of diabetes and their respective controls were pooled. Overall, our findings demonstrate a good correlation between ACE2 protein abundance and enzymatic activity indicating that the level of functional activity is highly dependent on the level of protein expression. In contrast, we found a lack of positive correlation between renal ACE2 activity and renal ACE2 mRNA levels in the *db/db* (Fig. 5*C*) as well as STZ-induced diabetic mice (Fig. 6*C*). This is in sharp contrast to the positive correlation between renal ACE2 activity and renal ACE2 protein (Figs. 5*D* and 6*D*). These findings illustrate the importance of not relying solely on mRNA levels when assessing whether ACE2 is altered. There are other emerging examples of translational regulation at the kidney level where mRNA is not affected. For instance, in renal epithelial cells, ANG II has been shown to increase vascular endothelial growth factor protein expression without altering mRNA levels (30,31). Measurements of ACE2 mRNA alone likewise may not reflect existing differences that occur at a posttranscriptional level. It is important to keep this in mind when interpreting data from studies reporting ACE2 gene polymorphisms in diseases such as hypertension or diabetes. Indeed, such studies have failed so far to reveal associations between different ACE2 genotypes and essential

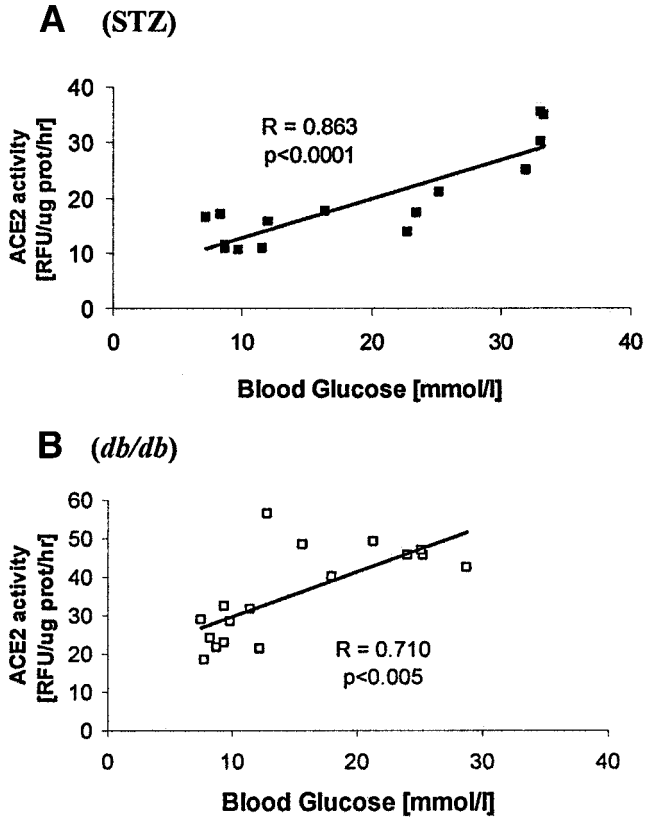

FIG. 7. Correlation between blood glucose and ACE2 activity in renal cortex tissue extracts from STZ-induced diabetic mice and their nondiabetic controls (*A*) as well as *db/m* and *db/db* mice (*B*).

hypertension (32) or diabetic nephropathy in patients with type 1 diabetes (33). Yet, it is possible that ACE2 may be altered in a tissue-specific manner at the level of ACE2 protein and enzymatic activity but remain unchanged at the gene level, as shown by our studies in two different models of diabetes (Fig. 4*A* and *B*). This further underscores the importance of having reliable assays to measure ACE2 activity in tissue samples.

Assays to measure ACE activity use spectrophotometric (24), fluorometric (34), high-performance liquid chromatography (35), or radiometric (36) approaches and are quite reliable, but there is limited information on ACE2 assays (28,29,37,38). We developed an assay to measure tissue ACE2 activity using 7-Mca-YVADAPK(Dnp) as a substrate. The dual cleavage of this substrate by ACE and ACE2 allowed us to measure the activity of these two carboxypeptidases concurrently. The fluorescence signal was partially quenched by specific inhibitors of both ACE and ACE2 but not by an inhibitor of another metalloprotease, carboxypeptidase A. The combination of specific inhibitors for ACE and ACE2 quenched the signal almost completely and to the same degree as EDTA, a metal ion chelator, indicating that both metalloenzymes, ACE and ACE2, are involved in the degradation of the substrate (Fig. 1).

ACE2 activity has been previously measured using a different fluorogenic substrate, (7-methoxycoumarin-4-yl) acetyl-Ala-Pro-Lys(2,4-dinitrophenyl)-OH (28,29,37). This approach detected ACE2 activity in serum samples from mice overexpressing ACE2, but not in the wild-type mice (29), as well as in rat heart tissue (28) and rat testes (37). Using this substrate in mouse tissues, however, we could not quench the fluorescence with MLN-4760 (data not shown). Moreover, the (7-methoxycoumarin-4-yl)acetyl-Ala-Pro-Lys(2,4-dinitrophenyl)-OH substrate is highly selective for ACE2 (29). Accordingly, it cannot be used for dual measurements of ACE and ACE2 activity. It should be noted that ACE2 activity can also be assessed from the formation of ANG I–VII from radioactively labeled ANG II (38). Since this method requires high-performance liquid chromatography measurements, the process involved is more complex.

The correlation between each respective ACE and ACE2 enzymatic activity and the relative abundance of each protein was highly significant over a wide range of protein expression in kidney extracts from ACE and ACE2 knockout and wild-type mice. In addition, we took advantage of having kidney cortex samples from ACE (16) and ACE2 (18) knockouts to validate our assay for enzymatic activity over a wide range of ACE and ACE2 protein levels in "spiking" experiments. These studies revealed a very close linear relationship between the amount of ACE and ACE2 added to the tissues from respective knockouts and their measured activities, respectively. Our studies in kidneys from ACE2 knockout mice revealed that the protein band that is active in terms of enzymatic activity is the 90-kDa band. This conclusion is based on the finding that the lack of this protein in the ACE2 knockout was associated with a level of enzymatic activity essentially undetectable (Fig. 3*B*). The 67-kDa ACE2 immunoreactive protein that is also abundantly present in the ACE2 knockout kidneys, by inference, does not appear to have detectable catalytic properties. Cell culture studies suggest, however, that an enzymatically active form of ACE2 of lower molecular weight than 90 kDa may exist in tissues other than kidney or in body fluids. For instance, in cultured astrocytes, the predominant ACE2 immunoreactive protein was detected at 70 kDa (39). The presence of this 70-kDa protein in conditioned media and its ability to convert ANG II to ANG I–VII suggests that this protein is a secreted form of ACE2 (39).

Our finding that ACE2 activity is altered in a tissue-specific manner in two different models of diabetes, the *db/db* and STZ-induced diabetic mice (Fig. 4*A* and *B*), deserves some comment. The mechanism for the tissue-specific changes in ACE2 activity is unknown, but it seems reasonable to speculate that it could be, directly or indirectly, a consequence of the hyperglycemic environment. This conclusion stems from our finding of a positive correlation between renal ACE2 activity and blood glucose and the fact that such a relationship was found in two different models of diabetes (Fig. 7). The fact that the upregulation of ACE2 is organ-specific (renal cortical tissue but not heart) argues, however, against a generalized effect of hyperglycemia on ACE2. The downregulation of ACE and upregulation of ACE2 in renal cortical tubules is an intriguing finding. Perhaps the high flow that occurs during hyperglycemia at the level of the renal tubules may be associated with local downregulation of the renin-ANG system in the proximal tubule, as expected from our finding of decreased ACE and increased ACE2. ANG II stimulates sodium reabsorption via activation of Na^+/H^+ exchange (40–42). Thus, one can view the downregulation of ACE and upregulation of ACE2 at the renal cortical tubular level as a possible mechanism to attenuate ANG II overactivity. This way, ANG II–driven proximal sodium reabsorption would be attenuated facilitating the sodium and water diuresis characteristic of uncontrolled hyperglycemia. It is of interest to note that in the glomerulus of diabetic mice, the opposite situation occurs (increased ACE in endothelial cells and decreased ACE2 in podocytes) (43,44). Thus, the impact of the hyperglycemic environment on these enzymes is not only tissue specific but cell specific as well (43,44). We postulate that a ratio of high ACE2 and low ACE favors less local ANG II accumulation. The finding of low ACE and high ACE2 in renal tubules from diabetic mice could explain renoprotection as reflected in the lack of tubulointerstitial involvement in young diabetic mice. By contrast, high ACE and low ACE2 in glomeruli from diabetic mice (43,44) may result in increased local levels of ANG II leading to altered glomerular permeability and albuminuria.

In summary, ACE2 is emerging as an important regulator in the pathway for ANG peptide degradation much in the same way that ACE is important in the formation of ANG II. The enzymatic assay that we have described in this report is unique in that it allows the simultaneous assessment of both ACE and ACE2 activity in tissue samples. This is particularly important because ACE2 and ACE colocalize in numerous tissues where both enzymes have been shown to be enzymatically active (4,5). Thus, the assessment of ACE2 activity, the only active homologue of ACE, in combination with ACE activity should help in the evaluation of the renin-ANG system in the pathophysiology of diabetic kidney disease. We conclude that in renal cortical tubules from diabetic mice, ACE2 is altered at the posttranscriptional level. Caution is therefore needed in interpreting data based solely on mRNA levels.

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