Localization of ACE2 in the renal vasculature: amplification by angiotensin II type 1 receptor blockade using telmisartan

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The Renin-Angiotensin System (RAS) plays a key role in the control of cardiovascular and renal function (8, 13, 30). Activation of the RAS has been widely incriminated in the pathophysiology of renal and cardiovascular diseases such as hypertension, myocardial infarction, and heart failure (21, 25). The RAS system primarily involves two enzymes: renin, which cleaves angiotensinogen to the inactive decapeptide angiotensin I (ANG I), and angiotensin-converting enzyme (ACE), a dipeptidyl carboxypeptidase that hydrolyzes ANG I to the octapeptide ANG II (7, 26). The discovery of angiotensin-converting enzyme (ACE)2, the only known enzymatically active homolog of ACE, has added a new level of complexity to the RAS (6, 9). ACE2 degrades ANG II to ANG-(1-7) and ANG I to ANG-(1-9) (10, 17). The impact of these actions of ACE2 and ACE on ANG II as well as the factors that directly or indirectly influence the activity of these enzymes need to be better defined. An understanding of the regulation of these enzymes is clinically relevant in view of recent studies showing that ACE2 expression is altered in pathological conditions such as diabetes, hypertension, and cardiovascular diseases (5, 27, 28, 31, 32, 34). The potential therapeutic effect of increased ACE2 activity has been recently recognized (2, 15, 22, 33).

ANG II type 1 receptor blockers are widely used as antihypertensive drugs that also decrease left ventricular hypertrophy and proteinuria (4, 18, 19). Vascular protection is critical to reduce cardiovascular and renal morbidity associated with diabetes and hypertension (12). The mechanism whereby ANG II blockade exerts its cardiorenal protective effect is not completely understood, but it is largely ascribed to blockade of actions such as vasoconstriction and inhibition of cell proliferation and fibrosis which are mediated by the ANG II type 1 receptor (24).

ACE2 is highly expressed in mouse kidney, where it has been localized in renal tubules and within glomerular epithelial cells (34). In glomeruli, both enzymes are localized in distinct cell types (34). The precise localization of ACE2 within the renal vasculature, to our knowledge, is unknown. In this study, we examined the localization of ACE2 in renal arterioles and hypothesized that the expression of this enzyme could be enhanced in vivo following blockade of the ANG II type 1 receptor. Enhanced ACE2 expression could counteract the reactive increase in ANG II that occurs after blockade of the ANG II type 1 receptor and this may help prevent excessive accumulation of ANG II within the vessel wall.

Materials and methods

Animal models. Female C57BLKS/J mice (8 wk of age) were used to identify the pattern of ACE and ACE2 distribution in the renal vasculature (Jackson Laboratory, Bar Harbor, ME). The mice were housed in cages (12:12-h light-dark cycle) with ad libitum access to chow and water. The Institutional Animal Care and Use Committee approved all procedures adhering to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. At 12 wk of age, mice (C57BLKS/J) were randomly assigned to drink either tap water (vehicle, n = 11) or tap water with telmisartan (kindly provided to us by Boehringer Ingelheim) at a dose of 2 mg·kg⁻¹·day⁻¹ (n = 11) for 2 wk.

After anesthesia using pentobarbital sodium injection, mice were killed by cervical dislocation. Kidneys were then removed and fixed in 10% paraformaldehyde. Kidneys were then removed and fixed in 10% paraformaldehyde.
The tail-cuff method was used to measure blood pressure. Blood pressure was measured in conscious-trained mice on 3 consecutive mornings using a PowerLab/S system connected to CHART software (A.D. Instruments, Milford, MA).

Immunohistochemistry. Paraffin blocks were cut at 4 μm and deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed with a pressure cooker at 120°C in target retrieval solution (DAKO). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Slides were incubated overnight at 4°C with rat monoclonal ACE antibody (5C4; a kind gift of Dr. S. M. Danilov; 1:800) or our ACE2 affinity-purified rabbit antibody (1:1,000), washed, and incubated with secondary antibody conjugated with peroxidase-labeled polymer (DAKO). After incubation with 3,3'-diaminobenzidine (DAB) chromogen, slides were counterstained with hematoxylin. Sections were dehydrated and covered with Permount (Fisher Scientific). Coverslips were viewed using a Zeiss microscope. To evaluate ACE2 and ACE protein expression in renal vessels, a semiquantitative analysis of the immunoperoxidase-stained sections was performed. Three blinded observers, previously trained by an experienced pathologist, identified 7–10 arterioles in each kidney section. Scoring of staining intensity was as follows: 1 = absent/weak staining, 2 = medium staining, and 3 = strong staining (27, 34). For statistical analysis, the data that were used were based on the percentage in the strong staining category only.

Immunofluorescence staining and confocal microscopy. Paraffin blocks were cut at 4 μm and mounted on superfrost plus slides (Fisher Scientific). Sections were rehydrated and antigen retrieved with a pressure cooker. For antigen colocalization, indirect immunofluorescence staining was performed. Sections were washed three times in PBS and permeabilized with 0.5% Triton X-100 for 5 min and blocked with 5% normal donkey serum in PBS for 1 h at room temperature. The sections were then incubated with primary antibodies including ACE rat 5C4 antibody (1:100) and ACE2 affinity-purified polyclonal rabbit antibody (1:200). Other antibodies used included ANG II rabbit polyclonal antibody (1:200; Peninsula Lab), ANG-(1-7) polyclonal antibody (1:200; a kind gift from Dr. R. A. S. Santos), PECAM-1 (CD31) goat antibody (Santa Cruz Biotechnology, 1:100), and anti-α-smooth muscle mouse antibody (Sigma, 1:100). Primary antibodies were diluted in 5% donkey serum in PBS (0.1% Tween-20 in PBS). Sections were washed three times in PBS and incubated with secondary antibodies diluted 1:200 in PBS with 5% donkey serum for 1 h at room temperature. For secondary antibodies, Alexa Fluor 488 (donkey anti-rat), Alexa Fluor 555 (donkey anti-rabbit), Alexa Fluor 647 (donkey anti-goat IgG), or Alexa Fluor 647 (donkey anti-mouse IgG) from Molecular Probes was used. After being washed three times with PBS, sections were mounted with Prolong Gold antifade reagent (Molecular Probes) to delay fluorescence quenching. After being covered with coverslips and sealed with nail polish, sections were visualized using Zeiss laser-scanning confocal microscope (LSM) 510 (Carl Zeiss Microscopy). Negative staining controls for the double or triple labeling procedures were performed by substitution of nonimmune serum for the primary antibodies.

Laser capture microdissection and real-time PCR. Frozen kidneys embedded in OCT medium were used to isolate arterioles from kidney cortex areas. Kidney frozen samples stored at −80°C were cut at 10 μm and stained with Histogène (Arcturus). Five or six arterioles were isolated from six sections of each mouse kidney using laser capture microdissection (LCM) with a phase microscope using a focused 30-μm laser beam (Arcturus). Renal arterioles were captured and collected onto LCM caps for mRNA extraction using an automated process. The mRNA was extracted using the PicoPure kit (Arcturus) and first-strand cDNA synthesis was performed using the cDNA reverse transcription Kit (TagMan). The cDNA was used undiluted for real-time PCR on an ABI Prims 7000 machine (Applied Biosystems). 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 ACE gene (GenBank no. BC026801): GGACCTCTACCTTCCTACATCACGC, CTACCCCCACATACCAACAGCA, and FAM-CAAAGTG-GATGCCCTCCTGGCCC-TAMRA and for ACE2 gene (GenBank no. BC040404): CAGAATCTACTCCACTGCGCAAGTG, TCGTGAG-GAACCCAGGATGT, and FAM-CAACAAAGACTGCCACCTGCTG-GTCC-TAMRA. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control (GenBank no. XM354601), which included forward primer, CAGAAGACTGTGAGGCCCTC; reverse primer, TGGCACCACATGCTTAG; and probe, FAM-CAGAAGACTGTGGACCCAGGATGT, and FAM-CAACAAAGACTGCCACCTGCTG-GTCC-TAMRA. 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.

Statistical analysis. Statistical analyses for comparison between groups were performed by an unpaired t-test and by Mann-Whitney U-test (SPSS version 12.0 for Windows). The latter nonparametric test was used when the sample size was small such as in the case of samples for ACE and ACE2 mRNA obtained by LCM. Statistical significance was defined when P < 0.05. Data are expressed as means ± SE computed from average results determined for tissue in each mice.

RESULTS

Immunolocalization studies. ACE and ACE2 were both found in kidney arterioles, but generally did not colocalize within the same vascular layer (Fig. 1). To localize ACE and ACE2, we used endothelial and smooth muscle cell markers. ACE strongly colo-

Figure 1. Angiotensin-converting enzyme (ACE) and ACE2 localization in renal vasculature: immunofluorescence staining of ACE (green; A) and ACE2 (red; B) in a kidney arteriole from mouse kidney. Merging both images show no colocalization of ACE and ACE2 (C). By contrast, neighboring tubules (see arrow) stain in yellow showing strong colocalization of ACE and ACE2.
ACE2 strongly colocalized with PECAM-1 (an endothelial cell marker), whereas ACE2 did not (Fig. 2).

ACE2 strongly colocalized with α-smooth muscle actin (SMA) in the vascular smooth muscle layer reflecting its presence in the vascular tunica media (Fig. 3, A, B, and C). ACE, by contrast, was localized in the vascular endothelial layer and was also present in the adventitia layer in the kidney arterioles (Fig. 3, D, E, and F).

ANG-(1-7) colocalized strongly with both PECAM-1 and with ACE (Fig. 4). This shows that ANG-(1-7) is localized in...
the endothelial layer (Fig. 4, A, B, and C). In addition, ANG-(1-7) also colocalized with α-SMA, reflecting its presence in the tunica media layer (Fig. 5, D, E, and F). ANG II colocalized strongly with PECAM-1 consistent with its presence in the endothelial cell layer (Fig. 5, A, B, and C). ANG II also colocalized with α-SMA reflecting its presence in the tunica media (Fig. 5, D, E, and F). The costaining between ANG II and α-SMA, however, was not as uniform compared with that seen between ANG-(1-7) and α-SMA.

**Effect of telmisartan on ACE2 and ACE immunostaining.** The administration of telmisartan for 2 wk resulted in a modest but significant decrease in blood pressure compared with vehicle-treated mice (systolic blood pressure 108.8 ± 4.7 vs. 97.8 ± 3.9, respectively; \( P < 0.05 \)). There were no significant
differences regarding body weight and kidney weight between vehicle- and telmisartan-treated mice (data not shown).

Immunohistochemical staining of kidney sections from 14-wk-old mice treated with telmisartan revealed an augmentation of ACE2 in kidney arterioles compared with untreated mice (Fig. 6, top). Enhanced staining was seen in arcuate and interlobular arteries. Semiquantitative analysis by three blinded observers (see MATERIALS AND METHODS) revealed that the percentage of vessels with strong ACE2 staining in the telmisartan group was increased compared with controls (57.1 ± 5.7% vs. 21.2 ± 6.3%, P < 0.001; Fig. 6). ACE in the endothelial layer in kidney vessels from telmisartan-treated mice, by contrast, showed decreased immunostaining compared with controls, but the difference did not reach statistical significance (32.5 ± 7.1% vs. 42.1 ± 10.1%, P = NS). Using the ACE/ACE2 ratio, however, there was a pronounced difference such that the ratio was markedly decreased in telmisartan-treated mice compared with controls (0.53 ± 0.14 vs. 7.59 ± 2.72, respectively, P < 0.05).

Effect of telmisartan on ACE and ACE2 mRNA in kidney arterioles isolated by LCM. LCM was used to isolate renal arterioles for mRNA analysis of ACE and ACE2 by real-time PCR. ACE2 mRNA expression was increased in kidney arterioles from mice treated with telmisartan, compared with mice treated with vehicle (1.80 ± 0.26 vs. 1.01 ± 0.09, respectively, P < 0.05; Fig. 7). ACE mRNA, by contrast, was decreased in renal arterioles from telmisartan-treated mice compared with controls, but the difference did not reach statistical significance (0.52 ± 0.17 vs. 1.06 ± 0.25, respectively, P = NS). Concordant with the immunostaining findings, the ACE/ACE2 mRNA ratio was significantly decreased in telmisartan-treated animals compared with controls (1.21 ± 0.31 vs. 4.63 ± 0.86, respectively, P < 0.05). The effect of telmisartan on the ACE/ACE2 ratio generated from immunostaining and mRNA, respectively, is summarized in Fig. 8.

DISCUSSION

This study shows that in renal arterioles from mouse kidney ACE2 predominantly colocalizes with α-SMA (a marker of smooth muscle cells), but not with PECAM-1, an endothelial cell marker. This was in sharp contrast to ACE, which strongly colocalizes with PECAM-1 but not with α-SMA. These find-
ings therefore show that in renal arterioles ACE2 is preferentially localized in the vascular tunica media layer, whereas ACE is localized in the endothelial layer. In a previous study, Zulli et al. (35) found ACE2 in \( /\text{H}9251 /\text{H} \)-SMA-positive cells, but this was reported in atherosclerotic plaques from thoracic aorta of male New Zealand white rabbits. Our study shows the localization of ACE2 within the tunica media layer of the renal vasculature but not in endothelium. Concordant with this finding in endothelium of renal arterioles, in a previous study ACE2 was also not detectable in mouse glomerular endothel-

Fig. 7. Top: representative example of a kidney arteriole from control mice before (A, left) and after laser microcapture (A, right). A, middle: isolated renal arteriole. B: summary of ACE2 mRNA expression in kidney arterioles from vehicle-treated (filled bars, \( n = 3 \)) and telmisartan-treated mice (open bars, \( n = 3 \)) showing increased expression of ACE2 in telmisartan-treated mice compared with vehicle-treated mice.

Fig. 8. A: ACE/ACE2 immunohistochemical staining ratio in kidney arterioles from telmisartan-treated mice (open bars) was markedly decreased compared with vehicle-treated mice (filled bars). *\( P < 0.05 \). B: ACE/ACE2 mRNA expression ratio in kidney arterioles from telmisartan-treated mice (open bars) was also significantly decreased compared with vehicle-treated mice (filled bars). *\( P < 0.05 \).
lum (34). It is interesting that ANG II, a main ACE2 substrate, and ANG-(1-7), the product of cleavage of ANG II by ACE2, are both present in the endothelial layer and tunica media. This suggests that degradation of ANG II to ANG-(1-7) by ACE2 takes place in the tunica media, whereas the formation of angiotensin by the ACE-dependent pathway takes place in the endothelial cells. Concordant with this view is the finding that ACE activity is high in serum, whereas ACE2 activity is barely detectable (2). ACE shedding from endothelial cells into the circulation has been documented (1).

We also found that after the administration of an ANG II type 1 (AT1) receptor blocker, telmisartan, there was an increase in ACE2 expression by immunostaining of renal arterioles. Concordant with this finding, ACE2 mRNA was also increased in renal arterioles isolated by laser microdissection. This suggests that a transcriptional mechanism is responsible for the increased ACE2 expression following chronic telmisartan administration. We speculate that enhanced ACE2 in the renal vasculature provides a mechanism of enhanced ANG II degradation that attenuates ANG II accumulation in the vessel wall following blockade of the AT1 receptor and the attendant reactive increase in ANG II levels (see below).

Interestingly, after telmisartan administration, the expression of ACE changed in the opposite direction than ACE2. That is, while ACE2 increased, ACE decreased after telmisartan administration. The effect of telmisartan administration on these enzymes was reflected on a marked decrease in the ACE/ACE2 ratio calculated from either immunostaining or mRNA data. Following telmisartan administration, an increase in ANG II levels is expected due to AT1 receptor blockade (14) as noted above. Our findings are consistent with the notion that such an increase is counterbalanced by an increase in ACE2, which favors ANG II degradation and a decrease in ACE, which would decrease its formation. Moreover, ACE not only promotes the formation of ANG II, but also the degradation of ANG-(1-7) (23). A decrease in ACE expression could therefore provide an additional vascular protective effect as a result of increased ANG-(1-7) levels. This would be the result of a decrease in its degradation owing to reduced ACE levels.

In this study, we did not measure either ANG II or ANG-(1-7) in the vessel wall since quantitative estimates of these peptides in the vessel wall, to our knowledge, are not technically possible. It is quite reasonable to postulate, however, that the observed decrease in the ACE/ACE2 ratio would lead to decreased ANG II formation (as a result of decreased ACE) and augmentation of ANG-(1-7) as a result of increased ANG II degradation (from increased ACE2). Such effects may complement the well-known effects directly related to the blockade of AT1 receptor by telmisartan. It should also be noted that telmisartan has an agonist peroxisome proliferator-activated receptor-γ activity that is unique among other ANG II blockers (3). Whether such an effect contributes, in part, to the observed changes in ACE2 and ACE needs to be investigated. Other ANG II receptor blockers can also increase ACE2 as shown in studies by Ferrario and others (11) in cardiac tissue showing that AT1 receptor blockade upregulates ACE2 expression. In addition, olmesartan administration increased ACE2 expression in the thoracic aorta from spontaneous hypertensive rats (14). This effect of olmesartan on ACE2 was not observed with other antihypertensive drugs such as atenolol and hydralazine (14). In a study by Whaley-Connell et al. (30) in the Ren2 transgenic rat, AT1 receptor blockade was associated with increased ACE2 expression. ACE inhibitors such as enalapril and lisinopril increased heart ACE2 expression in a model of experimental myocardial infarction after left coronary artery ligation and in the transgenic Ren-2 rats, respectively (16, 20). Blockade of either synthesis or activity using lisinopril and losartan, respectively, induced an increase in cardiac ACE2 activity (11). Our findings with telmisartan in the renal vasculature are in agreement with the aggregate of these observations in other tissues showing that inhibition of ANG II formation and blockade of the AT1 receptor result in ACE2 overexpression. It is also worthy to mention that in human kidney biopsies, the ACE/ACE2 ratio was increased in subjects with hypertension compared with controls (29). It is possible that part of antihypertensive action of ANG II blockers is related to lowering the ACE/ACE2 ratio, which would prevent ANG II accumulation within the renal vasculature.

In conclusion, our study shows that in renal arterioles ACE2 is localized in the tunica media, whereas ACE is localized in the endothelial layer. This suggests that these enzymes govern the degradation and formation of ANG II at these sites within the renal vasculature. Moreover, telmisartan, an ANG II type 1 receptor blocker, increases ACE2 expression in the tunica media of renal arterioles. This should facilitate the degradation of ANG II to ANG-(1-7) within the renal vasculature.

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References


