Expression of ACE and ACE2 in Patients with Hypertensive Nephrosclerosis

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Key Words
Hypertension · ACE2 · Renal disease

Abstract
Background: The interplay between intrarenal angiotensin-converting enzyme (ACE) and type 2 ACE (ACE2) might play important roles in the pathogenesis of hypertensive nephrosclerosis (HTN), but human data are limited. Methods: Renal biopsy specimens of 41 patients with HTN and 10 transplant donors as controls (CTL) were studied. The glomerular and tubulointerstitial mRNA expression of ACE and ACE2 was measured by laser microdissection and real-time quantitative polymerase chain reaction. The corresponding protein level was determined by immunohistochemistry. Results: Neither the glomerular nor tubulointerstitial mRNA expression of ACE or ACE2 correlated with the corresponding protein level by immunohistochemistry. The tubulointerstitial levels of ACE and ACE2 were significantly lower in HTN than CTL, while the glomerular ACE and ACE2 levels were similar between the groups. The tubulointerstitial ACE and ACE2 levels significantly correlated with the estimated glomerular filtration rate (GFR) and inversely with the degree of histological damage. The glomerular ACE and ACE2 levels significantly correlated with the rate of GFR decline. The ratio of glomerular ACE and ACE2 level correlated with the estimated GFR and the degree of glomerulosclerosis. Conclusion: Our results suggest that intrarenal ACE and ACE2 may play an important role in the pathogenesis and progression of HTN. Studies based on the mRNA expression of ACE and ACE2 should be cautiously interpreted.

Introduction
Hypertensive nephrosclerosis (HTN) is an important complication of systemic hypertension and a major cause of end-stage renal disease (ESRD) [1, 2]. However, the pathogenesis of HTN is not completely clarified. Studies have shown that blocking of the renin-angiotensin system (RAS) with angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) ameliorates renal injury and slows the disease progression of HTN, suggesting that RAS activation might play an important role in the pathogenesis of HTN [3–5]. In addition to increased intraglomerular pressure, intrarenal angiotensin II may directly increase the synthesis of growth factors and fibrogenic mediators, which also contribute to the hypertensive renal damage [6–8]. Direct evidence of the pathological relevance of intrarenal RAS in human HTN, however, is limited [9].

To complicate the picture, the newly-discovered type 2 ACE (ACE2) plays a regulatory role in RAS by cleaving...
anteriorin I to angiotensin 1–9 and angiotensin II to
anteriorin 1–7, counterbalancing the detrimental effect
of angiotensin II [10, 11]. It is highly possible that the
intrarenal activity of angiotensin II is regulated by the
interplay between ACE and ACE2. In the present study, we
explore the intrarenal mRNA expression and protein lev-
el of ACE and ACE2 in patients with biopsy-proven HTN.

Subjects and Methods

Subjects
We studied 41 consecutive patients in our hospital who had biopsy-proven HTN and no feature of other coexisting renal dis-
ases [12]. Renal biopsy tissue was retrieved for gene expression,
imunohistochemistry and morphometric analysis (see below).
Clinical data including blood pressure, serum creatinine, urea,
electrolyte, albumin, liver enzymes and 24 h urine protein were
reviewed. The glomerular filtration rate (GFR) was estimated by
a standard equation [13]. We also studied 10 kidney transplant
donors as controls (CTL).

Laser Microdissection
The method of laser microdissection has been described in our
previous study [14]. In essence, immediately after kidney biopsy,
part of the tissue was embedded by OCT compound (Sakura,
USA) and kept at –80 °C under a RNase-free condition for intra-
renal gene expression. We then performed laser microdissection
to determine the glomerular mRNA expression of ACE and
ACE2. Briefly, 8- to 10-μm thick cryosections were prepared on
a cryostat (Leica Microsystems, Wetzlar, Germany) using disposable
microtome blades (Leica Microsystems) under RNase-free
conditions. Sections were then mounted on glass slides covered
with a polyethylene naphthalate membrane (PALM Microlaser
Technologies, Bernried, Germany) and were treated with UV
irradiation and RNase Away (Invitrogen, Life Technologies, Pa.,
USA) prior to use. After being fixed in 70% ethanol for 2 min, the
sections were stained according to the following protocol: DEPC
water 1 min, Mayer’s hematoxylin (Sigma, USA) solution 30 s,
RNase-free water 1 min, 50% ethanol 1 min, 70% ethanol 1 min,
100% ethanol 1 min twice, and air-dried 10 min.

Next, PALM Microlaser Systems (PALM, Wolfratshausen,
Germany), which is equipped with a pulsed high-quality laser
beam, computer-controlled microscope stage and micromanipu-
lator was used to perform microdissection of the prepared slides.
Under direct visual control, 20 ± 5 glomerular areas of each case
were isolated by the focused nitrogen laser beam and then cata-
pulted into different microcentrifuge caps, which were filled with
guanidine thiocyanate containing lysis buffer (buffer RLT; Qia-
ergén, Inc., Canada). The tissue lysate of isolated glomerulus was
then stored at –80 °C until RNA extraction was performed.

Measurement of Intra-Renal Gene Expression
RNeasy Micro kits were used to extract total RNA from mi-
crodissected tissue according to the manufacturer’s protocol.
DNase was used to wipe off probable genomic DNA. For reverse
transcription, 5 μl total RNA was mixed with 1 μl random prim-
ers (150 ng), 1 μl dNTP mix (10 mM each), 4 μl 5x first-strand
buffer, 2 μl DTT (0.1 μ), 1 μl (200 U) Superscript II RNase H re-
verse transcriptase (all from Invitrogen, Life Technologies) and
made up to 20 μl with H2O. Reverse transcription was performed
at 56 °C for 5 min, 25 °C for 10 min, 42 °C for 50 min, and the in-
activated reaction at 70 °C for 10 min. The resulting cDNA was
stored at –80 °C until use.

We quantified intrarenal expression of ACE and ACE2 using
the ABI Prism 7700 Sequence Detection System (Applied Biosys-
tems, Foster City, Calif., USA). Commercially available TaqMan®
primers and probes, including two unlabeled PCR primers and
one FAM™ dye-labeled TaqMan MGB probe were used for all tar-
gent genes (all from Applied Biosystems). The primer and probe set
was deliberately designed across the intron-exon boundary so as
not to detect probable genomic DNA. For the real-time quantita-
tive polymerase chain reaction, 10 μl universal master mix, 1 μl
primer and probe set, 2 μl cDNA, and 7 μl H2O were mixed to
make a 20-μl reaction volume. Each sample was run in triplicate.
Time quantitative polymerase chain reactions were performed at
50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for
15 s and 60°C for 1 min. 18S rRNA (Applied Biosystems) was used
as a housekeeping gene to normalize the mRNA expression level
of each target gene. Results were analyzed with Sequence Detec-
tion Software version 1.7 (Applied Biosystems). In order to calcu-
late the differences of expression level for each target gene among
samples, the ΔΔCt method for relative quantitation was used ac-
cording to the manufacturer’s manual [15].

Immunohistochemistry and Morphometric Study of Kidney
Biopsy
The intrarenal ACE and ACE2 expression at protein level was
explored by a standard immunohistochemical technique. Briefly,
the renal tissue was fixed in 10% neutral buffered formaldehyde
overnight, followed by incubation with biotinylated secondar
antibody and demonstrated by peroxidase activity with per-
oxidase block for 5 min at room temperature. Sections
were then incubated in a serum block for 20 min at room temperature. Rabbit
polyclonal to ACE (catalog No. sc-20791; Santa Cruz Bio-
technology, Inc., Santa Cruz, Calif., USA) was then used for stain-
ing. Briefly, endogenous peroxidase activity was quenched by per-
oxidase block for 5 min at room temperature. Sections
were then counterstained with hematoxylin for 2 min. Sections
were dehydrated with graded alcohol and xylene, covered with
a coverslip.

As described in our previous studies [16, 17], Jones’ silver
staining was also performed on 5-μm thick sections of renal bi-
opsy specimens of each patient to assess renal scarring.

We used a computerized image analysis method to quantify
the degree of immunohistochemical and silver staining. Briefly, a
Leica TWIN Pro image analysis system (Leica Microsystems) was
connected to a Leica DMRXA2 microscope working with a 40× objective (final calculation: 0.258

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mm/pixel) and to a microcomputer for storage of the morphometric measurements and to perform image analysis by using image-analyzing software (MetaMorph 4.0; Universal Imaging Corp., Downingtown, Pa., USA). Ten glomeruli and ten randomly selected tubular interstitial areas were assessed in each patient and the average protein level and scarring degree in glomerular and tubulointerstitial areas, as represented by the percentage of the area with positive staining, were computed for each patient.

Clinical Follow-Up
All patients were followed for at least 12 months after renal biopsy. Their treatments were decided by an individual nephrologist and not affected by the study. Disease progression was measured by the rate of GFR decline [18], which was calculated by the least-squares regression method.

Statistical Analysis
Statistical analysis was performed by SPSS for Windows version 11.0 (SPSS, Inc., Chicago, Ill., USA). All results were presented as mean ± SD for normally distributed data and median (lower and upper quartiles) for the others. Since data of gene expression levels were highly skewed, nonparametric statistical methods were used. We used the Mann-Whitney U test to compare gene expression levels between groups and Spearman’s rank-order correlations to test associations between gene expression levels and clinical parameters. p < 0.05 was considered statistically significant. All probabilities were two-tailed.

Results
The demographic and baseline clinical data of the study subjects are summarized in table 1. Compared with kidney donors, hypertensive subjects were older, had a significantly higher blood pressure, more severe renal tissue damage and worse renal function. Amongst the HTN group, 23 patients (56.1%) were treated with ACE inhibitor, 2 (4.9%) with ARB, 19 (46.3%) with β-blocker, and 19 (46.3%) with calcium channel blocker.

Table 1. Demographic and baseline clinical data

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Patients</th>
<th>Kidney donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male:female</td>
<td>31:10</td>
<td>4:6</td>
</tr>
<tr>
<td>Age, years</td>
<td>57.04 ± 13.07</td>
<td>36.36 ± 13.73</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>143.66 ± 13.83</td>
<td>114.36 ± 14.20</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>81.83 ± 7.75</td>
<td>69.45 ± 9.77</td>
</tr>
<tr>
<td>Proteinuria, g/day</td>
<td>1.84 ± 2.91</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine, µmol/l</td>
<td>238.56 ± 234.36</td>
<td>82.73 ± 13.98</td>
</tr>
<tr>
<td>eGFR, ml/min/1.73 m²</td>
<td>41.88 ± 25.10</td>
<td>106.64 ± 22.03</td>
</tr>
<tr>
<td>Glomerulosclerosis, %</td>
<td>32.27 ± 24.98</td>
<td>2.63 ± 5.27</td>
</tr>
<tr>
<td>Tubulointerstitial fibrosis, %</td>
<td>33.66 ± 24.20</td>
<td>1.36 ± 2.34</td>
</tr>
</tbody>
</table>

Comparison of ACE and ACE2 Levels between Patients and Kidney Donors
The intrarenal mRNA expression levels of ACE and ACE2 in patients with HTN and kidney donors are summarized in figure 1. In brief, the glomerular mRNA expression levels of ACE were similar between HTN and CTL [ACE: 0.92 (0.00, 3.56) vs. 0.84 (0.00, 3.26), p = 0.923], while the glomerular ACE2 expression of HTN was marginally higher than that of CTL, although the difference fell short of statistical significance [3.58 (0.00, 14.74) vs. 1.16 (0.00, 1.87), p = 0.091]. The tubulointerstitial expression of ACE was marginally higher in HTN than CTL [2.28 (1.30, 4.45) vs. 1.21 (0.41, 1.91), p = 0.096]. In contrast, the tubulointerstitial expression of ACE2 was significantly higher in HTN than CTL [3.09 (1.46, 4.85) vs. 1.09 (0.44, 1.98), p = 0.042]. Subgroup analysis showed that the glomerular mRNA expression of ACE2 was marginally lower for patients who were receiving ACE inhibitor than for those who were not [2.33 (0.00, 3.48) vs. 7.94 (0.97, 23.73), p = 0.069], while the glomerul-
lar ACE expression and tubulointerstitial mRNA expression of ACE and ACE2 were similar between patients who were and were not receiving ACE inhibitor (details not shown).

The protein levels of ACE and ACE2 in HTN and CTL are quantified by immunohistochemistry (fig. 2) and summarized in figure 3. In brief, the glomerular ACE and ACE2 levels were similar between HTN and CTL (details not shown). In contrast, both the tubulointerstitial ACE and ACE2 levels were significantly lower in HTN than CTL [ACE: 5.31 (3.64, 7.27) vs. 10.50 (4.59, 14.43), p = 0.009; ACE2: 11.54 (7.85, 17.17) vs. 20.28 (18.88, 26.98), p = 0.001]. Both the glomerular and tubulointerstitial levels of ACE and ACE2 were similar between patients who were and were not receiving ACE inhibitor (details not shown).

**Correlations with Baseline Clinical Parameters**

There is no correlation between the ACE and ACE2 levels in glomerulus or tubulointerstitium and protein-
Correlations with Histological Damage

The degree of glomerulosclerosis had a modest but statistically significant correlation with the glomerular ACE2 level (r = −0.336, p = 0.039), but not with the glomerular ACE level (r = −0.068, p = 0.688) (fig. 5). The degree of glomerulosclerosis had a modest but statistically significant correlation with the glomerular ACE2 level (r = −0.336, p = 0.039), but not with the glomerular ACE level (r = −0.068, p = 0.688) (fig. 5).
degree of tubulointerstitial scarring, in contrast, significantly correlated with the tubulointerstitial ACE (r = –0.558, p < 0.001) and ACE2 levels (r = –0.643, p < 0.001) (fig. 5).

Correlations with Renal Function Decline Rate
The average duration of follow-up was 27.19 ± 9.29 months; the average rate of GFR decline was –4.06 ± 5.48 ml/min/year. The rate of GFR decline significantly correlated with the glomerular ACE (r = 0.582, p = 0.002) and ACE2 levels (r = 0.386, p = 0.046) (fig. 6), but not with the tubulointerstitial ACE (r = 0.186, p = 0.257) or ACE2 levels (r = 0.227, p = 0.165).

ACE/ACE2 Ratio
The ratio of glomerular ACE/ACE2 level correlated with the degree of glomerulosclerosis (r = 0.330, p = 0.046) and inversely with the estimated GFR (r = –0.385, p = 0.019) (fig. 7). On the other hand, the tubulointerstitial ACE/ACE2 ratio did not correlate with any clinical or histological parameter (details not shown).

Discussion
In the present study, we found a predominant presence of ACE and ACE2 in the tubulointerstitium. The tissue ACE and ACE2 levels were significantly decreased in patients with HTN; the magnitude of reduction correlated with the degree of renal failure and tubulointerstitial scarring. Notably, there was discordance between the protein level and mRNA expression of ACE or ACE2. We also found that the glomerular ACE and ACE2 level significantly correlated with the rate of renal function decline.
The intrarenal expression of ACE and ACE2 under pathological conditions has been extensively investigated in animal models, and the results were variable. ACE mRNA expression was reported to be unchanged [19, 20] or decreased [20, 21], and protein expression was reported to be decreased [21] in diabetic models. ACE2 mRNA expression was also reported to be unchanged [21, 22] or decreased [20], and protein expression was reported to be increased [22] in diabetic models, or decreased [23] in hypertensive models. Data on the intrarenal expression of ACE and ACE2 in humans is limited. In patients with diabetic nephropathy, ACE mRNA and protein expression was reported to be increased [20, 21]; ACE2 mRNA expression was reported to be unchanged and protein expression was reported to be decreased [24, 25]. This heterogeneity of study results is not well explained. It is, however, important to note that these studies investigated different species and animal models, and the duration or severity of nephropathy were highly variable. All these factors might cause this discordance in study results [25, 26]. Furthermore, different parts of renal tissue (i.e. glomeruli and tubulointerstitium) might be studied, whose expression of ACE and ACE2 might not be modulated synchronously [20, 25]. In the present study, we found that intrarenal expression of ACE and ACE2 was mainly in the tubulointerstitial area, which was consistent with previous studies [25, 27]. These results suggest that tubulointerstitial RAS might play an important role in the pathophysiology of HTN, which is similar to the observation in diabetic nephropathy [28]. On the other hand, we observed that the glomerular, but not the tubulointerstitial, ACE and ACE2 levels correlated with the rate of renal function decline, indicating that intraglomerular RAS may be relevant for the progression of HTN.

A previous study by Wakahara et al. [29] showed that the intrarenal expression is parallel in both ACE and ACE2. In line with this, we also found significant internal correlations between the glomerular as well as tubulointerstitial ACE and ACE2 at mRNA and protein levels (details not shown), and the ACE and ACE2 levels simultaneously correlated with clinical and histological parameters in the present study. At first glance, it seems paradoxical since ACE and ACE2 act antagonistically on the formation of angiotensin II. However, we further found that the ratio of glomerular ACE and ACE2 level correlated with the degree of glomerulosclerosis and inversely correlated with the estimated GFR. Although we did not measure the actual tissue level of angiotensin II, it is possible that a higher ACE/ACE2 ratio would result in an excess production of angiotensin II and aggravate renal injury. Taken together, these results suggest that it is the balance between ACE and ACE2 that governs the severity of renal injury in HTN.

Our results reconfirmed previous observations that the intrarenal expression of ACE and ACE2 might be modulated at the posttranscriptional level since mRNA and protein expression of ACE and ACE2 were not congruently regulated [21, 22]. In fact, we did not find any correlation between the mRNA expression and protein level of ACE or ACE2 in the present study, and it was the protein level rather than the mRNA expression of ACE or ACE2 that correlated with clinical or histological parameters (details not shown). Our results highlight the fact that data based on the intrarenal mRNA expression of ACE and ACE2 should be interpreted with caution.

We did not find any significant difference in the intrarenal ACE or ACE2, both in terms of mRNA expression or protein level, between patients who were taking ACE inhibitor or not. This observation may seem unexpected. However, it is well reported that the apparent drug tolerance in patients receiving chronic ACE inhibitor therapy is a result of the production of angiotensin II by alternative enzymes (e.g. chymase) rather than an alteration in the activity of ACE [30]. Since the number of patients who were taking ARB was small, subgroup analysis was not performed, and they were excluded from the analysis for the effect of ACE inhibitor.

There are several weaknesses of our study. First, since HTN is normally diagnosed clinically and renal biopsy is not a routine investigation in patients with essential hypertension, selection bias might exist in our study population, which possibly only included patients with atypical clinical characteristics and might be different from patients with ordinary essential hypertension that are commonly seen in daily clinical practice. Second, many of our patients were treated with some form of RAS-blocking therapy, which may influence the intrarenal expression of ACE or ACE2 [20, 31]. Thus, the results in the present study might reflect physiological response to treatment but not truly reflect changes in HTN. In theory, it would be interesting to measure the ACE and ACE2 activity in urine, which should reflect a renal source of the enzyme. Unfortunately, because of the limitation in the original design of our study, urine enzyme activity was not measured. Since other enzymes could contribute to angiotensin II and angiotensin 1–7 formation (e.g. chymase and neutral endopeptidase), it would also be interesting to explore their expression and relation with hypertensive renal damage in further studies. Finally, renal

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tissue from transplant donors was used as CTL in this study. However, there could be tissue injury in donor kidneys because of hemodynamic fluctuation and perfusing solution, and the intrarenal expression of ACE or ACE2 might change accordingly. In the present study, we did not test the specificity of the immunostaining because antibodies for both ACE and ACE2 were obtained from a commercial source, with specificity verified by the manufacturer by Western blotting and blocking experiments [32, 33], and they had been used with satisfactory results in previous studies [34, 35].

In summary, we found that the tubulointerstitial protein levels of ACE and ACE2 are decreased in patients with HTN, and the magnitude of reduction was related to the degree of renal failure and histological damage. On the other hand, the glomerular protein level of ACE and ACE2 correlated with the rate of renal function decline. We also found that there was discordance between the protein level and mRNA expression of renal ACE or ACE2. The results suggest that the balance between intrarenal ACE and ACE2 may play an important role in the pathogenesis and progression of HTN.

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Disclosure Statement

The authors have no conflicts of interest to declare.

References


