Human Urinary Kallikrein Can Generate Angiotensin II from Homologous Renin Substrates

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We previously proposed the “kinin-tensin system,” a unique vasoregulatory system that can produce both angiotensin II and kinins. To verify whether tissue kallikrein is a part of this system in humans, we examined the ability of human urinary kallikrein (HUK) to generate angiotensin (ANG) II directly from homologous renin substrates such as purified human angiotensinogen (AOGEN) and authentic human tridecapeptide renin substrate (13 RS). HUK released ANG II not only from ANG I but also directly from both AOGEN and 13RS at an optimum pH of 7.0. The amount of generated ANG II from 7.5 nmol of each of the three substrates at pH 7.0 was as follows: ANG I, 292.7 ± 67.2; 13 RS, 1951.7 ± 239.6; AOGEN, 2.2 ± 0.3 (pmol/3h, n=3 mean ± SE). HUK cleaved Phe-His and His-Leu bonds in 13 RS, and Tyr-Ile and Phe-His bonds in ANG I. These results suggest that HUK is a part of the “kinin-tensin system”, i.e., HUK can not only release kinins, but can also generate ANG II mainly through ANG I conversion and from AOGEN, the latter being a minor source of ANG II. Furthermore, HUK may play a role in regulating vascular tone under certain conditions in vivo. (Hypertens Res 1995; 18: 33-37)

Key Words: angiotensin II, kallikrein, renin substrate, angiotensinogen

Historically, the renin-angiotensin system has been regarded to be an endocrine system whose effects are exerted entirely through angiotensin II (ANG II) in the circulation. ANG II is the product of two proteolytic steps in the processing of the precursor, angiotensinogen. Renin produces the decapeptide ANG I which is biologically inactive. The subsequent cleavage of ANG I by angiotensin converting enzyme produces the active octapeptide ANG II. More recent evidence using molecular and biochemical approaches to angiotensin physiology raises the possibility that there are distinct, local renin angiotensin systems. Local renin angiotensin systems may act as paracrine and autocrine systems. Moreover, ANG II-generating systems such as tonin (1), trypsin (2), kallikrein (3), and cathepsin G (4), which are independent of angiotensin converting enzyme, have been demonstrated. More recently chymostatin-sensitive ANG II-generating enzyme (5) and chymase from the human heart (6) have been shown to convert ANG I to ANG II.

We previously demonstrated that porcine pancreatic kallikrein, which is known to generate lysylbradykinin from kininogen, has the ability to generate ANG II directly from human plasma protein (angiotensinogen). We proposed the term “kinin-tensin system” to refer to serine proteases such as kallikrein, tonin, and trypsin that act pH-depently, i.e., generate ANG II directly from angiotensinogen at weakly acidic pH (3, 7, 9), as well as generate kinins from kininogen at alkaline pH (8). Moreover, we also showed that tissue kallikrein purified from rat submandibular gland and human urine have the ability to convert ANG I to ANG II (10). Very recently, Krivoy et al. showed that tissue kallikrein from the porcine pancreas can generate ANG II from human plasma, which agrees with our results (11). However, evidence using human urinary kallikrein and human angiotensinogen has not yet been obtained.

In this study, we examined the ability of human urinary kallikrein (HUK) to produce ANG II directly from two different homologous renin substrates, i.e., synthetic tridecapeptide human renin substrate and purified human angiotensinogen (AOGEN), independent of renin or angiotensin converting enzyme. In addition, we compared the quantity of Ang II generated from three different substrates, i.e., Ang I, tridecapeptide renin substrate (13RS), and human AOGEN.

Materials and Methods

Chemicals
ANG I, ANG II, and human 13 RS were purchased from the Peptide Institute (Osaka, Japan).
Aprotinin was kindly provided by Bayer (Leverkusen, Germany). All other chemicals were of reagent grade. Purified HUK was generously provided by Sanwa Kagaku Kenkyusho Co., Ltd. (Nagoya, Japan) (12, 13). An amino acid sequence analysis (12 cycles) in our laboratory revealed that the sequence of this substance was compatible with that of the active form of HUK (14). Purified human AOGEN (19.1 μg ANG I/mg protein) was a generous gift from Dr. Duane A Tewksbury (15), Marshfield Foundation for Medical Research, Marshfield, WI, USA.

Proteins such as HUK and AOGEN were quantitated as protein by the method of Lowry et al. (16).

Determination of ANG II

Each of the substrates (ANG I, 13 RS, and AOGEN, concentrations shown in text) was incubated with HUK for 3 h in 0.2 M sodium phosphate buffer, at pH 6.0, 7.0, and 8.0 at 37°C in a total volume of 525 μl. The reactions were terminated by adding 6 volumes of ethanol, and the reaction mixtures were centrifuged. After evaporation of the supernatant, the residues were dissolved in 0.05 M sodium phosphate, and applied to a reverse phase HPLC column (Nucleosil 10 C 18, 0.4×20 cm) that had been pre-equilibrated with 20% acetonitrile in 0.05M sodium phosphate buffer (pH 5.5). The column was eluted isocratically for 5 min, and then with a linear gradient of acetonitrile (18% to 60% in 0.05 M sodium phosphate, pH 5.5) for 20 min at a flow rate of 2 ml/min. The eluate was monitored at 214 nm. When some fractions that contained ANG II were processed by a second elution, the column was eluted with a linear gradient of acetonitrile (10% to 75% in 0.1% TFA) for 25 min at a flow rate of 2 ml/min, and the eluate was monitored at 214 nm. The column was standardized with synthetic ANG I, ANG II, and 13 RS. Fractions that contained ANG II were processed by a second elution, the column was eluted with a linear gradient of acetonitrile (10% to 75% in 0.1% TFA) for 25 min at a flow rate of 2 ml/min, and the eluate was monitored at 214 nm. The column was standardized with synthetic ANG I, ANG II, and 13 RS. Fractions that contained ANG II or ANG I were collected and processed by RIA for ANG II and ANG I, as previously described (9). The recovery rate of ANG II was approximately 74%. For the inhibition experiments, aprotinin (10 μg/ml) was added to the incubation mixtures of HUK and each of the three substrates.

The cross-reactivities of the antiserum for ANG II with ANG I and 13 RS were 0.3% and 0.1%, respectively (9). Some of the ANG II fractions were processed for amino acid composition and sequence analysis.

Results

When 300 nmol of 13 RS and 30 μg of HUK were incubated for 3 h, two peaks (P-1a and P-1b) emerged on elution (Fig. 1A). When P-1a and P-1b were collected and subjected to a second elution on an HPLC column that had been equilibrated with 10% acetonitrile in 0.1% TFA, the retention time of the two peaks was reversed, and they were clearly separated (Fig. 2). The retention time of P-1a was identical to that of standard ANG II. Moreover, the amino acid composition and sequence of P-1a were identical to those of ANG II. On the other hand, the amino acid sequence of P-1b was Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His (ANG 1-9). In addition, ANG I RIA of fractions that had the same retention time as ANG I failed to detect ANG I. Thus, HUK released ANG II and Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His (ANG 1-9) directly from 13RS. Based on the mean area under the peak on HPLC in five experiments, HUK released ANG II and ANG1-9 from 13 RS in a ratio of approximately 3-to-1. The addition of aprotinin reduced the amount of both substances.

When ANG I (300 nmol) was incubated with HUK (30 μg), two peaks emerged (Fig. 1B). One peak (P-1) was eluted at the same time as synthetic ANG II. Amino acid sequence analysis revealed that this peak was identical to ANG II. The other peak (P-2) was shown to be Ile-His-Pro-Phe-His-Leu.

![Fig. 1. HPLC profiles of incubation mixtures of 13 RS and HUK (A), and ANG I and HUK (B). 300 nmol of 13 RS was incubated with 30 μg HUK for 3 h. When the incubation mixture was injected into an HPLC column that had been equilibrated with 20% acetonitrile in 0.05 M sodium phosphate buffer (pH 5.5), two peaks (P-1a and P-1b) emerged on elution (A). In the case of ANG I (300 nmol) and HUK (30 μg), P-1 and P-2 emerged. The retention time of P-1 was identical to that of standard ANG II (B). The amino acid sequence of P-2 was revealed to be Ile-His-Pro-Phe-His-Leu.](image-url)
Leu. ANG II fragments such as His-Leu and Asp-Arg-Val-Tyr (ANG I-4) were eluted nearly in void volume. Under other HPLC conditions two peaks emerged before that of ANG II and were demonstrated to be identical with these two substances by amino acid sequence analysis. Based on the mean area under the peak on HPLC in five experiments, HUK liberated ANG II and Asp-Arg-Val-Try (ANG I-4) in a ratio of 1-to-1. This reaction was inhibited by aprotinin.

When AOGEN (7.5 nmol) was incubated with HUK (30 μg) and passed through an HPLC column, the fractions that contained ANG II were processed by ANG II RIA. It was impossible to determine the amino acid composition and sequence since the amount available was too small. Serial dilutions of fractions that were eluted with the same retention time as synthetic ANG II were shown to be parallel to that of standard ANG II by ANG II RIA. However, ANG I RIA failed to detect ANG I in any fraction. When aprotinin was added to this reaction, immunoreactive Ang II was not detected.

The amount of ANG II generated (pH 7.0) from 7.5 nmol of these three substrates by 30 μg of HUK was as follows: ANG I, 292.7 ± 67.2; 13 RS, 1951.7 ± 239.6; AOGEN, 2.2 ± 0.3 (pmol/3h, n = 3 mean ± SE) (Table 1). The amount of generated ANG II was measured by RIA for ANG II after HPLC separation. With regard to the optimal pH of HUK for ANG II formation, the pH profiles of ANG II generation are also shown in Table 1. The optimal pH of HUK was closer to 7.0 than to 8.0. Judging from the amount of ANG II generated, the dose of each substrate was considered to be sufficient for this reaction.

Moreover, the amount of ANG II generated from these three substrates was dependent on the dose of each substrate (data not shown).

### Discussion

In this study, we demonstrated the ability of HUK to generate ANG II directly from human 13 RS and human AOGEN, as well as from ANG I. We previously showed that porcine pancreatic kallikrein and rat submandibular kallikrein could release ANG II directly from human plasma protein Cohn's fraction IV-4 under acidic conditions (3, 9), and that rat submandibular kallikrein and HUK also have the ability to convert ANG I to ANG II (10). More recently, Krivoy et al. independently demonstrated that tissue kallikrein from porcine pancreas can generate ANG II from human plasma using UV laser-desorption/ionization mass spectrometry (11). In this study, we showed that HUK cleaved Tyr-Ile and Phe-His bonds in ANG I, i.e., it converted ANG I to ANG II without further degradation; in addition, ANG II and Asp-Arg-Val-Try (ANG I-4) were liberated at a ratio of 1-to-1. In the case of 13 RS, HUK cleaved Phe-His and His-Leu bonds, and released Asp-Arg-Val-Try-Ile-His-Pro-Phe (ANG I-2) and Asp-Arg-Val-Try-Ile-His-Pro-Phe-His (ANG I-9) without further degradation from 13 RS in a ratio of approximately 3-to-1. Moreover, the present results also indicate that the optimal pH for ANG II formation from these three substrates by HUK is around 7.0, which agrees with our previous reports (9, 10).

The purity of HUK used in this experiment was confirmed by amino acid sequence analysis. Twelve cycles of amino acid sequence analysis revealed that the sequence of this substance was compatible with that of the active form of HUK, and no other peaks were detected. Furthermore, the formation of ANG II was inhibited by aprotinin.

Together, these results demonstrate that HUK has the ability to convert ANG I to ANG II, and to release ANG II directly from 13 RS and AOGEN.

In terms of the amount of ANG II generated, 13 RS appears to be the best of these three substrates.

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**Table 1. Generation of ANG II from 7.5 nmol of AOGEN, 13 RS, and ANG I at Various pH Levels**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH 6.0</th>
<th>pH 7.0</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOGEN</td>
<td>2.1±0.3</td>
<td>2.2±0.3</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>13 RS</td>
<td>1,165.7±75.2</td>
<td>1,951.7±239.6</td>
<td>1,322.7±259.3</td>
</tr>
<tr>
<td>ANG I</td>
<td>198.4±35.8</td>
<td>292.7±67.2</td>
<td>221.7±46.1</td>
</tr>
</tbody>
</table>

The amount of generated ANG II was expressed as pmol / 3h. (n = 3, mean±SE)
for ANG II formation by HUK. The rate of cleavage of AOGEN by HUK was approximately 500 times less than that of 13 RS at pH 7.0. In the case of kininogen, the specificity constant (Kcat/Km) for cleavage of the Met bond (N-terminal of the bradykinin moiety) by porcine pancreatic kallikrein is reportedly 3,500 times higher in kininogen than in the peptide Ser-Leu-Met-Lys-bradykinin, which has a partial kininogen sequence, and the Kcat/Km for hydrolysis of the Arg bond in the kininogen peptide Pro-Phe-Arg-Ser-Val-Gln is also 14 times lower than that for the release of lysyl-bradykinin from kininogen (17). Thus, kininogen is a better substrate for lysyl-bradykinin release by tissue kallikrein than a peptide with a partial kininogen sequence. Our results concerning substrate specificity for the release of ANG II from AOGEN by tissue kallikrein are in striking contrast to those for the release of lysyl-bradykinin from kininogen.

Chymase from the human heart was recently identified and characterized as a serine protease. This enzyme has a very high specificity for the conversion of ANG I to ANG II (18). In contrast, HUK generated ANG II more efficiently from 13 RS than from ANG I. Therefore, the substrate specificity of HUK is different from that of human chymase. Moreover, HUK cleaved Tyr-Ile and Phe-His bonds in the ANG I sequence. In this regard, HUK is similar to neutrophil cathepsin G, mast cell chymase, and chymotrypsin (19).

With regard to the biological significance of ANG II formation by tissue kallikrein, the present results suggest that tissue kallikrein might release ANG II directly from AOGEN, independently of renin and angiotensin converting enzyme, under certain conditions in vivo. In fact, we previously showed that aprotinin, nafamostat (a serine protease inhibitor) and chymostatin (a cysteine protease inhibitor), but not captopril (an angiotensin converting enzyme inhibitor), suppressed intracardiac ANG II formation during myocardial ischemia in nephrectomized dogs, i.e., in the absence of the renal angiotensin system, and suggested that both serine protease(s) and chymostatin-inhibitable protease(s) release ANG II (20, 21). An intrinsic angiotensin system was recently demonstrated in the canine pancreas. Interestingly, ANG II and AOGEN, but not ANG I or renin, were demonstrated in the canine pancreas, which suggests that ANG II is generated directly from AOGEN by kallikrein (22). In some human tissues, tissue kallikrein might contribute to the formation of ANG II independently of renin and angiotensin converting enzyme.

In addition, we also demonstrated that nafamostat is effective for improving blood flow in patients with peripheral vascular disease, probably by inhibiting the local formation of ANG II by serine protease(s) (23). Not only in such a pathological condition as the above peripheral vascular disease, in normal subjects nafamostat inhibited ANG II formation in our recent study (24). These findings led us to the hypothesis that ANG II may be locally formed directly from AOGEN or from ANG I by serine proteases such as tissue kallikrein under certain conditions in humans. Considering the amount of ANG II generated from the three substrates, 13 RS is the best substrate for HUK to generate ANG II. However, 13 RS is not a natural substrate. From a physiological perspective, tissue kallikrein might play a role in regulating vascular tone by ANG I conversion.

In summary, we have shown that HUK, similar to porcine pancreatic kallikrein, as we previously reported, can generate ANG II from human AOGEN and human 13 RS, as well as from ANG I. The biological significance of ANG II formation by tissue kallikrein requires further investigation.

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References


