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Big angiotensin-25: A novel glycosylated angiotensin-related peptide isolated from human urine

Sayaka Nagata [a], Kinta Hatakeyama [b], Maki Asami [a], Mariko Tokashiki [a], Hajime Hibino [c], Yuji Nishiuchi [c,d], Kenji Kuwasako [e], Johji Kato [e], Yujiro Asada [b], Kazuo Kitamura [a,*]

[a] Circulatory and Body Fluid Regulation, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki 889-1692, Japan
[b] Pathology, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki 889-1692, Japan
[c] SAITO Research Center, Peptide Institute, Inc., Ibaraki, Osaka 567-0085, Japan
[d] Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan
[e] Frontier Science Research Center, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki, Miyazaki 889-1692, Japan

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ABSTRACT

The renin–angiotensin system (RAS), including angiotensin II (Ang II), plays an important role in the regulation of blood pressure and body fluid balance. Consequently, the RAS has emerged as a key target for treatment of kidney and cardiovascular disease. In a search for bioactive peptides using an antibody against the N-terminal portion of Ang II, we identified and characterized a novel angiotensin-related peptide from human urine as a major molecular form. We named the peptide Big angiotensin-25 (Bang-25) because it consists of 25 amino acids with a glycosyl chain and added cysteine. Bang-25 is rapidly cleaved by chymase to Ang II, but is resistant to cleavage by renin. The peptide is abundant in human urine and is present in a wide range of organs and tissues. In particular, immunostaining of Bang-25 in the kidney is specifically localized to podocytes. Although the physiological function of Bang-25 remains uncertain, our findings suggest it is processed from angiotensinogen and may represent an alternative, renin-independent path for Ang II synthesis in tissue.

1. Introduction

The renin–angiotensin system (RAS) plays key roles in the regulation of blood pressure and electrolyte and body fluid dynamics. According to the classical concept, the systemic RAS consists of renin, angiotensinogen (Aogen), angiotensin-converting enzyme (ACE), angiotensin (Ang) I, Ang II, and the Ang II type 1 and 2 receptors. Upon activation of the systemic RAS, renin is secreted from the juxtaglomerular apparatus in the kidneys into the circulation, where it acts on Aogen secreted from the liver to produce Ang I, which is in turn converted to Ang II by ACE [1,2]. Ang II then binds to Ang II type 1 and 2 receptors to exert its biological effects. In addition, it is now recognized that a wide variety of tissues and organs, including the heart, vasculature, kidney and nervous system, among others, produce Ang II, which then acts in an autocrine/paracrine fashion independently of the systemic RAS [3,4]. Within the heart, for example, local Ang II appears to contribute to both the maintenance of myocardial homeostasis and to adaptive responses induced by cardiac stress, such as those caused by prolonged hypertension. Elevated levels of cardiac Ang II activity are also associated with diseases of the heart and vasculature, including cardiac hypertrophy, coronary artery disease and myocardial infarction. Similarly, excess local Ang II in the kidney is associated with glomerular sclerosis, diabetic nephropathy and renal arterial stenosis.

Over the past 20 years, novel components of the RAS, including the (pro)renin receptor, ACE2, Ang (1–7), Ang III and Ang IV, as well as their receptors, have been identified and studied [5–8]. To examine the biosynthesis of Ang II within tissue, we developed a radioimmunoassay (RIA) that recognizes the N-terminal sequence of Ang II. Then using that assay, we isolated and characterized proangiotensin-12 (proang-12), as a major molecular form in rat small intestine [9]. Proang-12 is an angiotensin-related peptide with the same amino acid sequence as Ang I plus two additional amino acids. When measuring tissue levels of proang-12 and Ang II in rats treated with RAS inhibitors or fed a low-salt diet, we found that the tissue levels of proang-12 and Ang II did not correspond to the circulating RAS activity. Instead, Proang-12 appears to be an important intermediate involved in the regulation of rat tissue Ang II [10,11].

Up to now similar angiotensin-related peptides had never been reported in humans. In the present study, however, we describe an angiotensin-related peptide isolated from human urine and assess the potential function of the novel peptide.

* Corresponding author. Address: Circulatory and Body Fluid Regulation, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan. Fax: +81 985 85 6596.
E-mail address: kazuokit@med.miyazaki-u.ac.jp (K. Kitamura).

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2. Materials and methods

2.1. Ethics statement

Urine samples were collected from healthy volunteers. Tissue samples were obtained from the Miyazaki University Hospital. Written informed consent was obtained from all patients, and the study protocol was approved by the institutional review board (No. 817).

2.2. Peptides and enzymes

Ang I and II were purchased from Peptide Institute Inc. (Osaka, Japan) and Ang II + Cys was synthesized by Bex Co., Ltd. (Tokyo, Japan). Renin and chymase substrate were synthesized by Peptide Institute Inc. (Osaka, Japan). Aogen was purchased from Calbiochem. Renin and mast cell chymase were purchased from AnaSpec, Inc. (Fremont, CA, USA) and Elastin Products Company, Inc. (Owensville, MO, USA), respectively.

2.3. Radioimmunoassay for the N-terminal portion of Ang II

The radioimmunoassay (RIA) for the N-terminal portion of Ang II was performed using the method described previously [9]. The standard buffer was 50 mM sodium phosphate (pH 7.4) containing 0.5% bovine serum albumin (BSA), 0.5% Triton X-I00, 80 mM NaCI, 25 mM EDTA–2Na, 0.05% NaN_3 and 500 KIU/ml of aprotinin. The RIA incubation mixture was composed of 100 μl of standard Ang II or the sample to be determined, 50 μl of antiserum against the N-terminal of Ang II at a dilution of 1:5000, and 50 μl of ^{125}I-labeled ligand (18,000 cpm). After incubation for 24 h, the reaction was stopped by adding 50 μl of 1% γ-globulin and 200 μl of 23% polyethyleneglycol (#6000) in the standard buffer. After vigorous shaking, the mixture was incubated at 4 °C for 15 min and centrifuged at 2000×*g* for 30 min. The radioactivity in the resultant pellet was measured in a gamma counter (Aloka ARC-600, Tokyo). All assay procedures were performed in duplicate at 4 °C, as with the RIA for the C-terminal region of adrenomedullin [12]. This RIA cross-reacted with Ang I and Ang III at levels of 50% and 12.5%, respectively, but did cross-react with Ang IV or Ang (1–7).

2.4. Purification procedure

After collecting 5.5 L of urine from three healthy subjects, the urine was applied to a Sep-Pak C18 cartridge (35 ml, Waters) and eluted with 60% acetonitrile in 0.1% trifluoroacetic acid. The eluted sample was lyophilized, dissolved in 10 mM NH_4COOH (pH 4.0), and then applied to a CM 52 column (2.5 × 75 cm). After washing the column with 10 mM NH_4COOH (pH 4.0), the fraction containing immunoreactivity was eluted with 100 mM NH_4COOH (pH 4.0). The eluate was concentrated and subjected to gel filtration on a Sephadex G-50 column (3.0 × 150 cm), after which the fraction showing immunoreactivity was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on a diphenyl column (0.46 × 25 cm, Grace Vydac Inc., Deerfield, IL, USA). The fraction with immunoreactivity was then further purified on an affinity column (Affi-Gel 10 Active Ester Agarose; Bio Rad, Tokyo, Japan), which had been prepared with anti-Ang II N-terminal antiserum. Finally, the purified peptide was subjected to RP-HPLC using an ODS-120A column (0.46 × 15 cm, Tosho, Tokyo, Japan). During these purification steps, levels of immunoreactive Ang II N-terminal were monitored using RIA described previously [9].

2.5. Structural determination

To determine the amino acid sequence and molecular weight of the purified peptide, a tandem mass spectrometric analysis was performed using a positive electrospray ionization with a QSTAR Elite Hybrid LC/MS/MS System (AB SCIEX, USA), and the amino acid sequence was determined using a Procise 494 HT Protein Sequencing System (Applied Biosystems, USA). For mass spectroscopy, the sample was dissolved in a solution of 50% acetonitrile in 0.1% formic acid. To characterize the glycosyl chain, the conditions used for the enzymatic release, fluorescent labeling, separation and structural identification of N-glycan were the same as reported previously [13,14]. Briefly, the delipidated extract was subjected to proteolysis catalyzed by pepsin plus glycoamidase A, and the resultant peptides were further digested to amino acids using pronase. After purification on a Bio-Gel P-2 column, the reducing ends of the released N-glycans were labeled with fluorescent 2-aminopyridine, and any excess reagents were then removed by Sephadex G-15 chromatography. The pyrimidylamino (PA)-glycans were then separated based on charge on a TSK-gel diethylaminoethyl (DEAE)-5PW column (Tosoh, Tokyo, Japan), after which each separated fraction was applied to a Shim-pack HRC octadecyl silica (ODS) column (Shimadzu, Kyoto, Japan) for separation based on hydrophobicity, and the elution time of each peak was expressed as a glucose unit (GU) value. The molecular mass of each PA-glycan fraction was then analyzed using Maldi time-of-flight mass spectrometry (MALDI-TOF-MS). Fractions containing two types of glycan were further subfractionated based on molecular size using a TSK-gel Amide-80 column (Tosoh), and the elution positions were calibrated in GU values. Sample PA-glycans were mapped on the basis of their GU and molecular mass values, and their coordinates were compared with those of reference PA-glycans in the GALAXY database.

2.6. Renin and chymase kinetics

Human Bang-25 and NonG-Bang-25 were synthesized by Peptide Institute, Inc. (Osaka, Japan). Renin substrates (Bang-25, NonG-Bang-25, Aogen) at a concentration of 1–200 μM were incubated with 25 ng of recombinant human renin for 0, 30 or 60 min at 37 °C in 0.5 M PBS buffer (pH 7.4) containing 0.02% BSA. The reaction was stopped by addition of 4 volumes of acetic acid and boiling for 5 min. Ang I was then quantified using the previously described RIA for Ang I [9].

Chymase substrates (Bang-25, NonG-Bang-25, Aogen) at a concentration of 1–200 μM were incubated with 10.85 mU of mast cell chymase for 0, 30 or 60 min at 25 °C in 0.6 M Tris–HCl + 3 M NaCl (pH 8.0). The reaction was stopped by addition of 4 volumes of HPLC Buffer A (10% CH_3CN, 0.1% trifluoroacetic acid) and boiling for 5 min. Ang II was quantified using the aforementioned RIA for Ang II [9].

2.7. Immunohistochemical staining of human Bang-25

Human Bang-25 was detected using antiserum raised in rabbit against the C-terminal portion of the peptide. Thereafter, the polyclonal antibody was affinity purified by Scrum Inc. (Tokyo, Japan) using Bang-25-(18–25) as the antigen. For subsequent immunohistochemical staining, formalin-fixed, paraffin-embedded tissue blocks were cut into 4-μm-thick sections and labeled using the anti-Bang-25 antibody (100× dilution in PBS). As a negative control, the antibody was replaced with non-immune rabbit serum (Dako Japan, Inc.). Then after blocking endogenous peroxidase activity using hydrogen peroxide, the sections were incubated with an EnVision+/HRP System (Dako Japan, Inc.), and the staining was developed using 3,3′-diaminobenzidine. Finally the sections were counterstained with Meyer's hematoxylin.

2.8. Statistical analysis

Values are presented as means ± S.E. Comparisons of enzyme kinetics data were made using analysis of variance (ANOVA) followed by Bonferroni's test. Values of $P < 0.05$ were considered significant.

3. Results and discussion

When we screened human urine gel filtration fractions for peptides using an antibody raised against the N-terminal portion of Ang II, we found a highly immunoreactive peptide with an apparent molecular weight of about 5000 (Fig. 1A). Notably, this peptide was present at concentrations estimated to be more than 100 times higher than those of Ang I and Ang II.

We next purified this peptide from 5.5 L of human urine using ion-exchange, gel-filtration and affinity chromatography steps, and then finally RP-HPLC, which yielded a single peak (Fig. 1B). With this protocol we obtained 200 pmol of purified peptide from the 5.5 L of urine. When the sample of purified peptide was subjected to mass spectrometry, we found a hexahydric charged ion with $m/z = 780.18$, a pentavalent ion with $m/z = 936.02$, and a quadrivalent ion with $m/z = 1169.76$ (Fig. 1C), which collectively indicate the molecular weight of the purified peptide to be 4675.1. The amino-acid sequence of the peptide was then determined to be DRVYIHPFHLVIHX^{1}ESTX^{2}EQLAKAN (X, not identified), and from the known amino acid sequence of Aogen [15], X^1 and X^2 were identified as Asn and Cys, respectively (Fig. 1D). In addition, because we found a MS/MS peak for a glycosyl chain, the purified peptide was digested with N-glycosidase and again subjected to mass spectrometry. Under these conditions, the pentavalent ion had a $m/z = 611.71$ and the quadrivalent ion had a $m/z = 764.39$ (data not shown), which indicates the molecular weight of the deglycosylated peptide to be 3053.6 and confirms that X^2 is Cys–Cys.

Structural analysis of the glycosyl chain was delegated to GLYENCE Co., Ltd. After using diethylaminoethyl (DEAE) and ODS column chromatography to purify the glycosyl to a single peak (Fig. 1E), the resultant ODS fraction was subjected to MALDI-TOF-MS glycoanalysis using the three MS axes and the chromatography database (GALAXY) [14,16]. The results showed the peptide in this fraction to have a molecular mass of 1720 Da and to give rise to an oligosaccharide of 9.8 (ODS) and 6.9 (DEAE) GU, which corresponds to reference PA-glycan code No. 200.4 in

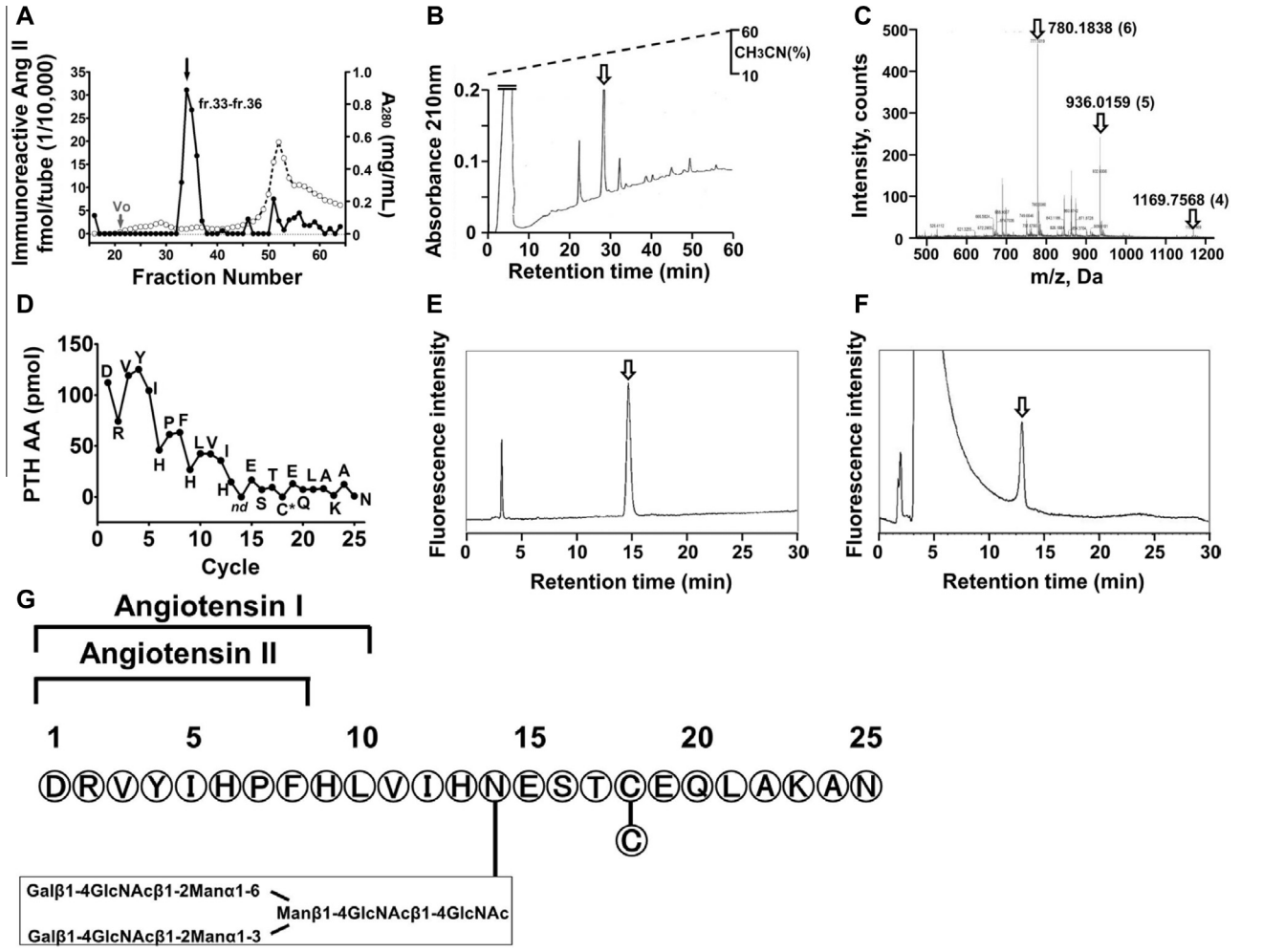

Fig. 1. (A) Immunoreactive Ang II N-terminal in human urine. Extract of human urine was subjected gel filtration (Sephadex G50), and the immunoreactive Ang II N-terminal in each fraction was assayed using a specific RIA: closed circles, immunoreactive Ang II N-terminal (fmol/tube); open circles, absorbance at 280 nm (mg/ml). The arrow indicates the highly immunoreactive Ang II N-terminal fraction. (B) Final purification by RP-HPLC. An elution gradient of 10–60% CH_3CN in 0.1% trifluoroacetic acid was run for over 60 min at a flow rate of 1 ml/min. (**C**) Determination of the molecular weight of the purified peptide based on quadrupole time-of-flight (Q-TOF) mass spectrometry. (D) Amino acid sequence analysis using a protein sequencer; 25 amino acid residues were detected. (E) Primary structural analysis of the glycosyl chain. A single peak was detected. (F) Secondary structural analysis of the glycosyl chain. Both the candidate (arrow) and GALAXY glycosyl chains were run on an ODS column. (G) Structure of human Bang-25 isolated from urine. GlcNAc: *N*-acetylglucosamine, Gal: galactose, Man: mannose.

the GALAXY database. The identity of this sample was then confirmed using co-chromatography (Fig. 1F) and MALDI-TOF-MS analyses, which showed the glycosyl chain to be a complex sugar chain consisting of nine sugars, which is often found with N-linked glycosylation. The complete structure of the molecule is shown in Fig. 1G. We are calling this novel peptide Big angiotensin-25 (Bang-25), because it consists of a 25-amino acid peptide that is N-glycosylated on Asn14 and has a cysteine linked to Cys18. Human Aogen contains four putative asparagine-linked glycosylation sites (Asn14, 137, 271, 295) and contains four cysteines (Cys18, 138, 232, 308) [15,17–19], with Cys18 and Cys138 linked by a disulfide bridge. From its structure it appears clear that Bang-25 is a derivative of Aogen.

Knowing the structure, we synthesized Bang-25 using a combination of solid phase and liquid phase techniques, which will be described elsewhere. The synthetic peptide was confirmed by amino acid analysis, MALDI-TOF-MS analysis and RP-HPLC. With the RP-HPLC, for example, native and synthetic Bang-25 emerged at the exact same elution position.

Using the synthetic peptide, we found that renin processed Bang-25 into Ang I, but the rate was slow (Fig. 2A and C). By contrast, chymase rapidly digested Bang-25 to produce Ang II (Fig. 2B and D). When we compared Aogen, Bang-25 and nonglycosylated Bang-25 (NonG-Bang-25), each at a concentration of 1 μM, as substrates for renin and chymase ($n = 6$ for each reaction), we found that renin rapidly produced Ang I from Aogen, but production of Ang I from Bang-25 was much slower (Fig. 2A). Consistent with that result, the K_m for Aogen was estimated to be 1.2 μM, which is comparable to the value reported previously [17,19], while the K_m and V_{max} for Bang-25 were 95 μM and 340 pmol/min/ml (Fig. 2C). In addition, the K_m and V_{max} for NonG-Bang-25 were 24 μM and 307 pmol/min/ml (Fig. 2C). It therefore seems doubtful that Bang-25 functions as a substrate for renin in vivo. Moreover, renin catalyzed significantly greater production of Ang I from NonG-Bang-25 than from Bang-25, indicating the glycosyl chain protects the peptide from renin (Fig. 2A and C). This is consistent with the earlier observation that N-linked glycosylation on Asn14 is an important inhibitor of binding to renin. Still, renin does not cleave NonG-Bang-25 as efficiently as it cleaves Aogen. Given that the Aogen N-terminus is linked to the rest of the molecule by a disulfide bridge between Cys-18 and Cys-138, which plays a key role in the peptide's binding to renin [19] and is absent from Bang-25, it is not surprising that Bang-25 is cleaved less efficiently by renin than is Aogen, with or without glycosylation.

Chymase, by contrast, does not act on human Aogen (Fig. 2B) [20], but it efficiently digests both Bang-25 and NonG-Bang-25 (Fig. 2B and D). The K_m and V_{max} were 9.6 μM and 129 pmol/min/ml for Bang-25 and 7.5 μM and 155 pmol/min/ml for NonG-Bang-25 (Fig. 2D). And although production of Ang II from NonG-Bang-25 tended to be greater than from Bang-25, the difference was not statistically significant in the present experiment (Fig. 2D). This suggests Bang-25 functions as a substrate for chymase, which is consistent with our finding that the tissue Ang II concentration was much higher than the Ang I concentration.

We then assessed the distribution of Bang-25 in tissues by immunostaining with an antiserum raised against the C-terminal portion of the peptide. We found that Bang-25 was abundantly expressed in a number of human tissues, including kidney (Fig. 3G and H), heart (Fig. 3C), adrenal gland (Fig. 3A), pancreas (Fig. 3B) and placenta (Fig. 3D–F), among others (Table 1). For practical reasons, we used the placenta as an exemplar for further study. There we found Bang-25 to be localized to extravillous trophoblasts. The

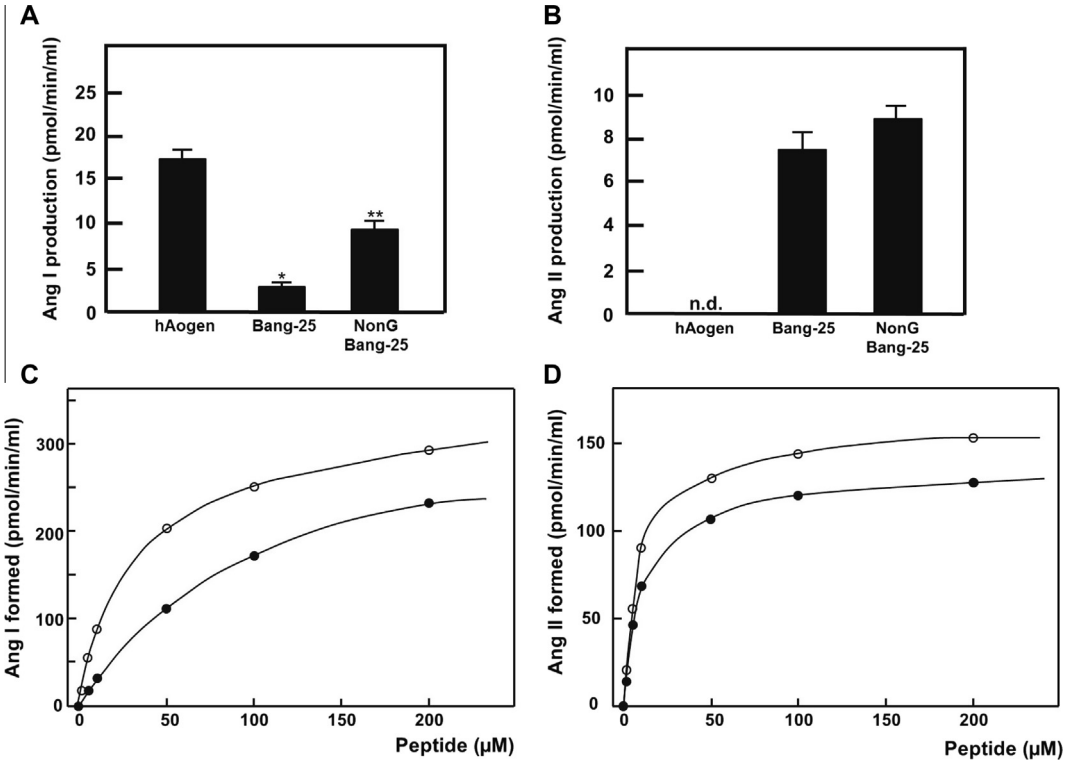

Fig. 2. (A) Velocity of renin-catalyzed Ang I formation at a substrate concentration of 1 μM ($n = 6$). The results are shown as means ± SEM. $^{*}P < 0.05$ vs. NonG-Bang-25 and $P < 0.01$ vs. hAogen. $^{**}P < 0.01$ vs. hAogen. (B) Velocity of chymase-catalyzed Ang II formation at a substrate concentration of 1 μM ($n = 6$). The results are shown as means ± SEM. (C) Michaelis–Menten plots for the renin-catalyzed release of Ang I from Bang-25 (closed circles) and NonG-Bang-25 (open circles) ($n = 2$–6). The K_m and V_{max} were 95 μM and 340 pmol/min/ml for Bang-25 and 24 μM and 307 pmol/min/ml for NonG-Bang-25. (D) Michaelis–Menten plots for the chymase-catalyzed release of Ang II from Bang-25 (closed circles) and NonG-Bang-25 (open circles) ($n = 2$–6). The K_m and V_{max} were 9.6 μM and 129 pmol/min/ml for Bang-25 and 7.5 μM and 155 pmol/min/ml for NonG-Bang-25.

placenta was also highly immunoreactive to our anti-Ang II N-terminal antibody, and gel filtration showed the molecular weight of the antigen to be approximately 5000, just as in human urine (Fig. 3I). Moreover, although gel filtration produced a clear Ang II peak, there was little Ang I peak, indicating Ang II was produced via a renin-independent pathway.

The abundance of Bang-25 over such a wide range of tissues suggests it is important in the production of Ang II in tissue. Particularly interesting to us was the strong Bang-25 staining in endocrine tissues, including pancreatic cells and the adrenal gland medulla. It has been reported that Ang II contributes to oxidative stress, inflammation and apoptosis in pancreatic cells [21], and that local Ang II may stimulate catecholamine release from the adrenal medulla [22]. Within that context, the possible conversion of Bang-25 to Ang II in tissue suggests Bang-25 may indirectly contribute to the regulation of endocrine cell function through Ang II production. Alternatively, the observation that it is highly localized in endocrine cells suggests Bang-25 may itself function as an endocrine hormone without conversion to Ang II. In the kidney, Bang-25 is localized predominantly to podocytes (Fig. 3G and H). This is noteworthy, as it is known that pathological production of Ang II by podocytes causes injury [23] related to such disease states as primary glomerulopathy, hypertension and diabetes mellitus [24], and reduced podocyte loss can entirely account for the renoprotective effect of Ang II blockade [25]. Although it is not presently clear whether podocytes are the origin of Bang-25 in urine, it could be that Bang-25 in urine is marker of podocyte injury.

Chymase, the enzyme that cleaves Bang-25 to produce Ang II, is released into interstitial tissues from mast cell granules following tissue injury and during inflammation, and chymase expression is upregulated after kidney injury and in heart disease [26]. In human vascular extracts, chymase inhibition reduces Ang II synthesis by more than 90% [27], suggesting chymase-dependent Ang II synthesis is stimulated by tissue injury or inflammation. One possibility is that, in tissue, Aogen is subject to cleavage by a different enzyme to form Bang-25, which is in turn cleaved by chymase to form Ang II.

Table 1
Result of the immunohistochemistory.

Kidney	Podocyte	++
Heart	Left ventricle, cardiocytes	++
Lung	Pulmonary artery, SMCs	+
	Bronchiole, epithelium	+
Esophagus	Stratified squamous epithelium (basal layer)	+
Stomach	Glands (endocrine cells)	+
	Nerve (submucosal and Auerbach)	+
Large intestine	Crypts (endocrine cells)	+
Liver	Hepatocytes	+
Spleen	Histiocytes	+
Pancreas	Islets	++
	Exocrine (acinus/duct)	–
Adrenal gland	Medulla	++
	Cortex	–
Lymph nodes	Histiocytes	+
	Dendritic cells	+
Testis	Sertoli cells	++
Salpinx	SMCs	++
	Tubal epithelium	+
Placenta	Extravilous trophoblasts	++
	Villi	–

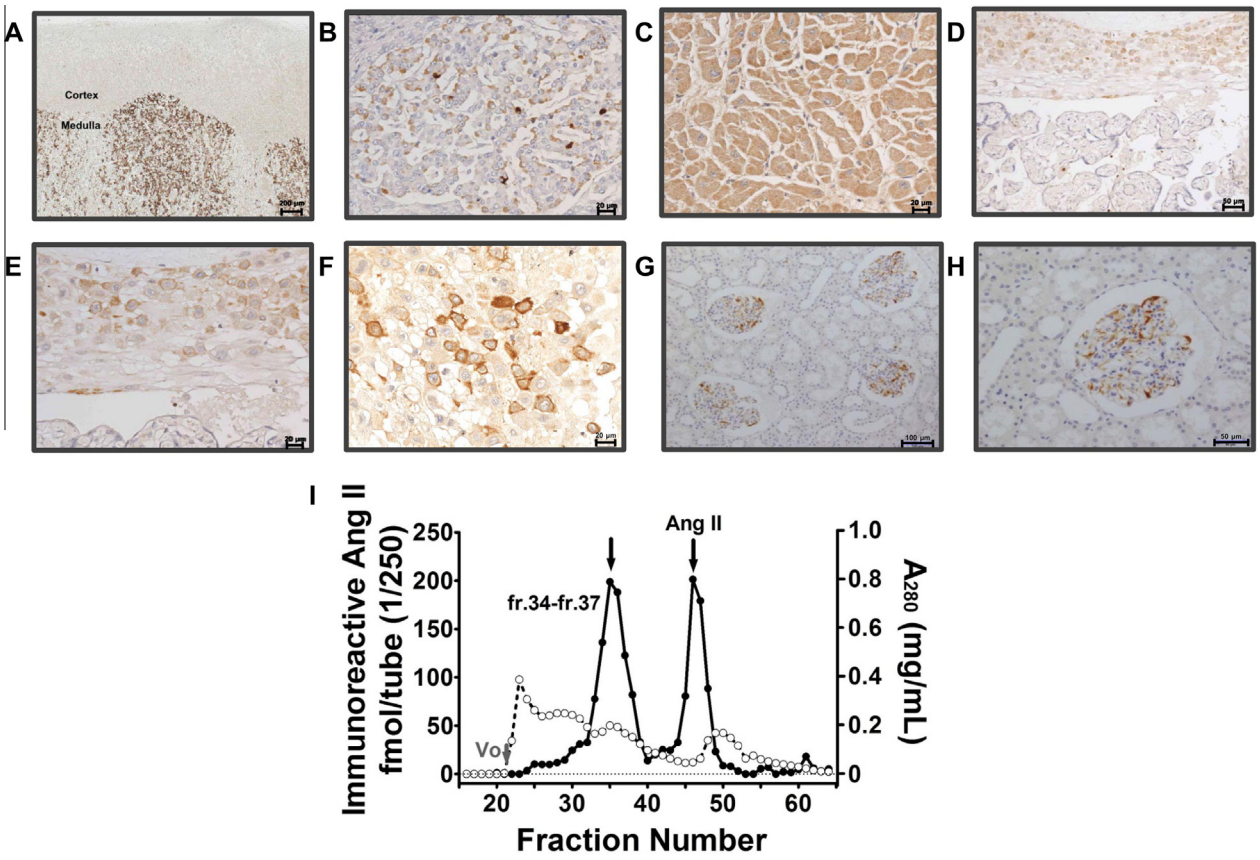

Fig. 3. (A–H) Immunohistochemical staining of Bang-25 in representative sections of human tissue. Bang-25 immunostaining in adrenal gland (A), pancreas (islet cells; B), heart (C), placenta (D–F) and kidney (G and H). The staining in kidney was localized to podocytes (G and H). (I) Immunoreactive Ang II N-terminal in placenta. An extract of human placenta was subjected gel filtration (Sephadex G50), and the immunoreactive Ang II N-terminal in each fraction was assayed using a specific RIA: closed circles, Ang II N-terminal (fmol/tube); open circles, absorbance at 280 nm (mg/ml).

In the present study, we isolated and identified a novel Aogen-derived peptide, Bang-25, which is composed of 25 amino acids, is N-glycosylated on its 14th amino acid (Asn), and has a cysteine linked to its 18th amino acid (Cys). The identification of Bang-25 suggests the existence of a RAS processing cascade different from the renin-catalyzed cleavage of Aogen to Ang I, and provides a potential target for assessing Ang II in tissue and for the development of new therapeutic approaches to related diseases.

Acknowledgments

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