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

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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.

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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.