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Isolation and sequence determination of human brain natriuretic peptide in human atrium

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We isolated human brain natriuretic peptide (human BNP) from the human atrium. Sequence analysis has revealed that it is a 32-amino-acid peptide with the sequence S-P-K-M-V-Q-G-S-G-C-F-G-R-K-M-D-R-I-S-S-S-S-G-L-G-C-K-V-L-R-R-H, which is identical to the C-terminal sequence (77–108) of the human BNP precursor deduced from the cDNA sequence. The sequence of human BNP (77–108) is preceded by Pro^{75}-Arg^{76} in the human BNP precursor, which is the same processing signal as Pro^{97}-Arg^{98} of the precursor of atrial natriuretic peptide (ANP). The processing of the BNP precursor occurs in the cardiocyte, although that of the ANP precursor in the cardiocyte is unclear at present.

Natriuretic peptide, brain; Natriuretic peptide, atrial; Processing; (Human atrium)

1. INTRODUCTION

Since the discovery of porcine brain natriuretic peptide (BNP), accumulating evidence indicates that a family of natriuretic peptides are involved in the regulation of water-electrolyte balance and blood pressure [1]. Although specific radioimmunoassays (RIAs) for porcine BNP were established, no porcine BNP-like immunoreactivity (-LI) has been detected in the heart or in the brain of rats, humans, or monkeys [2]. These results indicate that, unlike atrial natriuretic peptide (ANP), BNP is heterogeneous in the amino acid sequence among species.

Recently, we have isolated rat BNP from rat atrial tissues using an antiserum against the ring structure of iso-ANP [3,4] and demonstrated that the amino acid sequence of rat BNP is composed of 45 amino acid residues [4,5]. Flynn et al. [6] also reported the isolation of iso-ANP from the rat atrium but the sequence of iso-ANP reported differs from that of rat BNP we reported [4,5]. In the course of the isolation of rat BNP, Matsuo and his colleagues reported the cDNA sequences encoding the precursors for human BNP [7] and rat BNP [8]. The C-terminal 45-amino-acid sequence of the rat BNP precursor is identical with the sequence of rat BNP [4,5].

As for human BNP, we have established a specific RIA for human BNP using synthetic human BNP-26 or human BNP(83–108) based on the nucleotide sequence of the human BNP precursor [7] and demonstrated that the major form of human BNP in the atrium is a low-molecular-weight form of BNP comigrating with human BNP-32 in reverse-phase high performance liquid chromatography (RP-HPLC) (Mukoyama et al., unpublished observations).

In the present study we have isolated human BNP from the human atrium using a specific RIA for human BNP and determined its sequence.

2. MATERIALS AND METHODS

2.1. *Materials*

Human BNP(77–108), or human BNP-32 was synthesized by the solid-phase method. α-Human ANP, or ANP(99–126) was synthesized by the conventional solution methods [9]. The atrial tissue was obtained at autopsy from a patient without cardiac complications.

2.2. *RIA*

RIA for human BNP used in the present study is reported elsewhere. The cross-reactivity of α-human ANP in the RIA for human BNP was less than 0.01% on a molar basis. RIA for ANP was performed according to the previous report [10]. The cross-reactivity of human BNP-32 in the RIA for ANP was less than 0.01%.

2.3. *Isolation*

The atrium (50 g) was boiled in 1 M acetic acid (500 ml) for 10 min. It was homogenized with a Polytron homogenizer for 10 min and centrifuged at 15000 rpm for 60 min at 4°C and the supernatant was then loaded on an SP-Sephadex C-25 column (Pharmacia, Sweden;

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50 mm i.d. × 100 mm). After being washed with 1 M acetic acid, the adsorbed material was eluted with 2 M pyridine (SP-II) and 2 M pyridine-acetate (pH 5) (SP-III) successively. The SP-III fraction was directly treated with Sep-Pak C_{18} cartridges (Waters, USA). The fraction passing through the cartridges was diluted twice with 0.1% trifluoroacetic acid (TFA) and was applied again on other Sep-Pak C_{18} cartridges. The eluate with 60% acetonitrile containing 0.1% TFA was lyophilized. The resulting material was applied on a Sephadex G-50 column (Pharmacia, Sweden; 24 mm i.d. × 410 mm, 1 M acetic acid). Two-ml fractions were collected and aliquots were subjected to RIA for human BNP. Immunoreactive fractions (nos. 43–50) were concentrated with a Sep-Pak C_{18} cartridge. The adsorbed material was then applied on a TSK-gel CM-2SW column (Toyo Soda, Japan; 4 mm i.d. × 250 mm) and eluted with 10% acetonitrile–0.05% Triton X-100–ammonium formate (pH 7.4) in a linear gradient from 10 mM to 1.0 M. Immunoreactive fractions were purified on a Nucleosil $5C_{18}$ column (4.6 mm i.d. × 150 mm; Nagel) with a linear gradient of acetonitrile from 15–30% in 0.1% TFA. Further purification was performed by use of a Vydac diphenyl column (4.6 mm i.d. × 250 mm; Vydac) as described previously [4].

2.4. *Lysyl-endopeptidase digestion*

One μg of the isolated peptide was subjected to reductive carboxymethylation and subsequent lysyl endopeptidase digestion according to the procedure described previously [4]. The digest was applied on a Nucleosil $5C_{18}$ column (4.6 mm i.d. × 150 mm). Four fragments, designated L1, L2, L3 and L4 (fig.4), were separated by gradient elution with acetonitrile from 0 to 50% in 0.1% TFA.

2.5. *Sequence analysis*

Three hundred ng of the isolated peptide and the peptide fragments, L1, L2, L3 and L4 were subjected to sequence analysis. It was carried out by stepwise Edman degradation using a gas-phase sequencer equipped with a RP-HPLC system, Model 477A/120A (Applied Biosystems Inc.).

2.6. *Amino acid analysis*

Isolated peptide (100 ng) was hydrolyzed with 6 M HCl at 100°C for 24 h. The resulting amino acids were converted to phenylthiocarbamyl derivatives and then analyzed on RP-HPLC according to Ebert [11] with some modifications.

3. RESULTS

3.1. *Identification of human BNP in HPLC*

The tissue concentrations of human BNP and ANP in the human atrium were estimated to be 0.39 μg/g and 7.85 μg/g, respectively. Fig.1 shows the elution profiles of BNP-LI and ANP-LI of the crude extract of RP-HPLC. The major peak with human BNP-LI emerged at 22 min, which was the same retention time as that of synthetic human BNP-32. On the other hand, ANP-LI eluted at the same elution position as that of the ANP precursor, γ-human ANP.

3.2. *Isolation of human BNP*

On chromatography using SP-Sephadex C-25, about 10 μg of BNP-LI was detected in the SP-III fraction. In gel-permeation chromatography using Sephadex G-50 (superfine) of the SP-III fraction, BNP-LI appeared in the low-molecular-weight region (fig.2). Further purification on HPLC using TSK-gel CM-2SW (carboxymethylated silica gel; a cation exchanger) and Nucleosil $5C_{18}$ (octadecyl silica gel) afforded 4 μg of BNP-LI. The purified peptide was obtained by HPLC with a Vydac diphenyl column (fig.3). The final yield was estimated to be 1.5 μg of BNP-LI (8%).

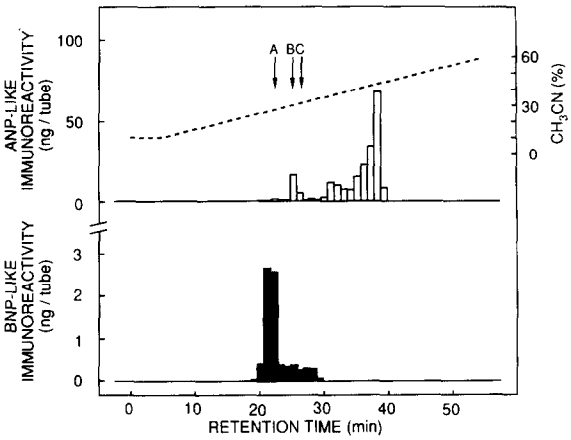

Fig.1. Elution profile of atrial extract in RP-HPLC. A Nucleosil $5C_{18}$ column (4.6 mm i.d. × 150 mm) was used. Elution was performed with a linear gradient (1%/min) of acetonitrile from 10% to 60% in 0.1% TFA. ANP-LI and BNP-LI are expressed with α-human ANP and human BNP-32 used as standards, respectively. A–C indicate the elution positions of synthetic human BNP-32, α-human ANP and its dimer, respectively.

3.3. *Sequence determination of human BNP*

The amino acid composition of the isolated peptide is shown in table 1. Sequence analysis of the isolated peptide revealed that the N-terminal sequence is S-P-K-M-V-Q-G-S-G-X-F-G-R-K-M-D-R-I-S-S-X-X-G-L-X-X-X-V-L-R, where X expresses an unidentified residue. In order to elucidate the complete sequence, the intact peptide was exposed to reductive carboxymethylation and to subsequent enzymatic digestion with lysyl endo-

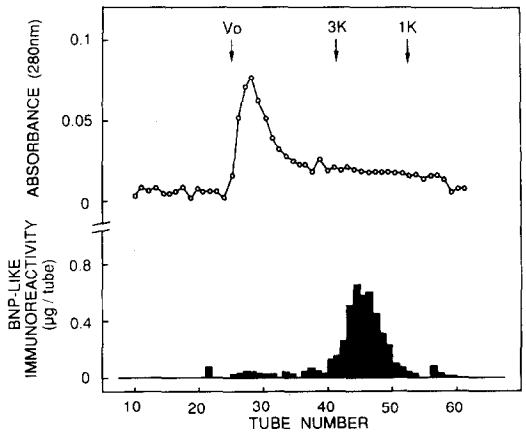

Fig.2. Gel-permeation chromatography of SP-III fraction, Vo, 3K and 1K indicate elution positions of bovine thyroglobulin, α-human ANP and caerulein, respectively.

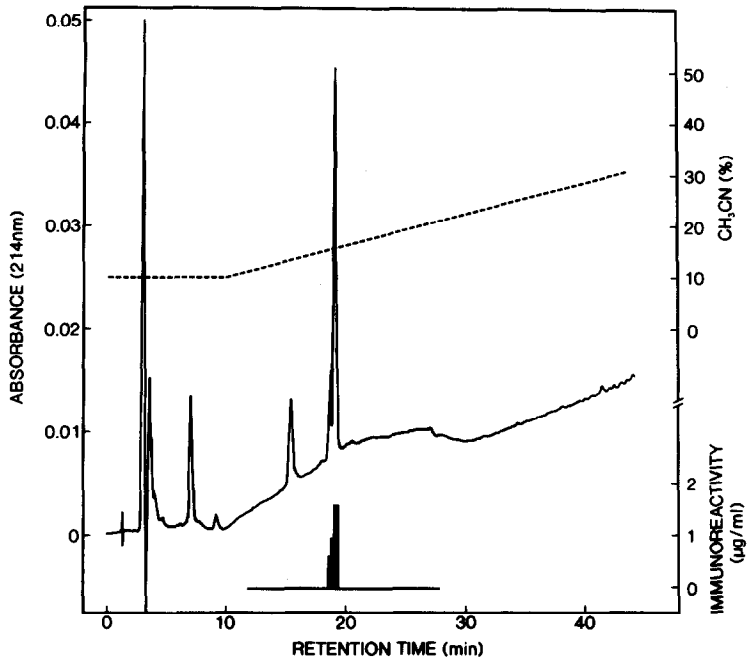

Fig.3. RP-HPLC of semi-purified immunoreactive material. A Vydac diphenyl column was used. Elution was performed as described in section 2.

peptidase. As shown in fig.4, four fragments designated L1, L2, L3 and L4 were obtained. These fragments proved to be S-P-K, V-L-R-R-H, M-V-Q-G-S-G-C-F-G-R-K and M-D-R-I-S-S-S-S-G-L-G-C-K, respectively, by sequence analysis of the individual peptide fragments. These results on amino acid analysis and sequence analysis indicate that human BNP isolated from the human atrium is S-P-K-M-V-Q-G-S-G-C-F-G-R-K-M-D-R-I-S-S-S-S-G-L-G-C-K-V-L-R-R-H or human BNP(77–108) as illustrated in fig.5.

Table 1

Amino acid ratio of the isolated peptide

Amino acid	Ratio	Deduced ratio[a]
Asx	0.9	1
Glx	0.7	1
Ser	5.7	6
Gly	5.5	5
His	0.9	1
Arg	4.0	4
Pro	0.9	1
Val	2.0	2
Ile	1.0	1
Leu	2.0	2
Phe	1.1	1
Lys	3.0	3
Met	0.9[b]	2
$(Cys)_2$	n.d.[c]	1

[a] Calculated from the cDNA data [7].
[b] Methionine was recovered as its sulfoxide because hydrolysis was carried out under usual conditions as described in section 2. The low recovery may be due to degradation on acid hydrolysis.
[c] Not determined.

4. DISCUSSION

In the present study, we have isolated human BNP from the human atrium and demonstrated that human BNP is a 32-amino-acid peptide, which corresponds to the C-terminal sequence (77–108) of the human BNP precursor deduced from the cDNA sequence [7]. The sequence of human BNP(77–108), or human BNP-32 is preceded by Pro^{75}-Arg^{76} in the human BNP precursor [7], which is the same processing signal as that of the ANP precursor [12]. The accumulated evidence indicates that ANP is stored as its precursor or γ-ANP (ANP(1–126)) in the heart and that it is secreted from the heart as α-ANP or ANP(99–126), which is generated by the processing at the Pro^{97}-Arg^{98} sequence of γ-ANP on secretion [13,14]. In contrast, the present study clearly shows that the major storage form of BNP in the human atrium is not the human BNP precursor but human BNP-32, suggesting that the processing of the BNP precursor at the signal of Pro^{75}-Arg^{76} occurs in the human atrium. Further studies are necessary to elucidate the mechanism of the preferential processing of the BNP precursor in the human heart.

Porcine BNP is stored as a large molecule, BNP(1–106), in the heart [15,16] and is present as BNP-26 and BNP-32 in the brain [1,17]. Rat BNP in the rat heart exists mainly as a 45-amino-acid peptide, which is secreted into the circulation [3–5]. In addition, bovine BNP, or ASIF (aldosterone secretion inhibitory factor) which was isolated from bovine chromaffin cells is known to be a 35-amino-acid peptide, of which C-terminal 28-amino-acid sequence is identical with that

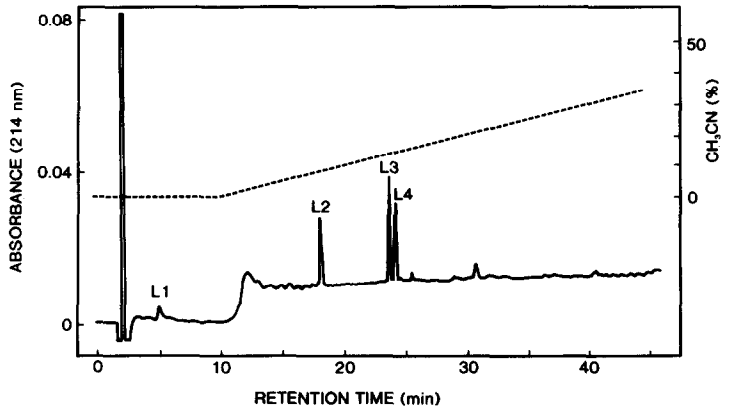

Fig.4. RP-HPLC of lysyl-endopeptidase digests of carboxymethylated human BNP.

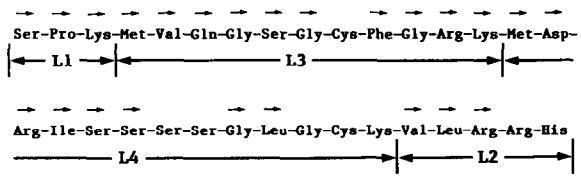

Fig.5. Amino acid sequence of human BNP. Arrows indicate the residues determined by sequence analysis of the native peptide.

of porcine BNP-32 [18]. These previous observations and the finding on human BNP in the present study indicate that the molecular form of BNP varies markedly among species unlike ANP.

Recently, two types of human receptors for natriuretic peptides, ANP-A and ANP-B receptors, possessing the guanylate cyclase domain were determined by molecular cloning [19–21]. In these studies, it was reported that the ANP-B receptor is preferentially activated by porcine BNP rather than α-human ANP, whereas the ANP-A receptor responds similarly to both natriuretic peptides. Therefore, the sequence determination of human BNP achieved in the present study opens up the new research field in the signal transduction system of natriuretic peptides, ANP and BNP, in humans.

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