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ISOLATION AND IDENTIFICATION OF A HIGH MOLECULAR WEIGHT BRAIN NATRIURETIC PEPTIDE IN PORCINE CARDIAC ATRIUM

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SUMMARY: Brain natriuretic peptide (BNP)-like immunoreactivity has recently been shown to exist in porcine heart, although this peptide was first identified in porcine brain. A high molecular weight (MW) BNP was isolated from cardiac atrium by using anti-BNP immunoaffinity chromatography and reverse phase high performance liquid chromatography. By microsequence analyses of the native high MW BNP as well as its tryptic and chymotryptic peptides, the complete amino acid sequence of the high MW BNP was determined. The high MW BNP consisted of 106 amino acid residues including one disulfide linkage and carried a BNP structure at its C-terminus. Since the high MW BNP was found to correspond to the gamma-form (storage form in heart) of the ANP family, this high MW BNP was designated "gamma-BNP".

Brain natriuretic peptide (BNP) is a novel type of natriuretic peptide recently identified in porcine brain. BNP has amino acid sequences and pharmacological effects strikingly similar to those of atrial natriuretic peptide (ANP) (1). So far BNP of 26 residues (BNP-26) and its N-terminally extended form (BNP-32) had been identified as major endogenous forms of BNP in the brain (1-3). By utilizing a radioimmunoassay specific to BNP, we screened immunoreactive (ir-) BNP in porcine central nervous system and peripheral tissue, and found the highest concentration of ir-BNP in the cardiac atrium (3,4). Characterization of ir-BNP in cardiac atrium demonstrated that the majority of ir-BNP existed as a high MW form of 12,000 daltons on gel filtration in a manner similar to that observed for the ANP family. Moreover, this high MW ir-BNP emerged as a single component on reverse phase high performance liquid chromatgraphy (HPLC) (4). These results suggested that BNP was mainly present in porcine cardiocyte as a γ-form. Based on recently obtained data, we have isolated γ-BNP from the cardiac atrium and have determined its amino acid sequence, as reported in the present paper.

MATERIALS AND METHODS

Isolation: Porcine hearts were collected from a local slaughter house soon after killing. Cardiac atria (100 g from 5 pigs), mainly containing auricles, were dissected from whole hearts. Diced tissue was boiled for 10 min in 10 volumes of water to inactivate intrinsic proteases. After cooling, glacial

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acetic acid and HCl were added (final concentrations = 1M and 20mM, respectively), and the resulting mixture was homogenized with a Polytron mixer for 5 min. The supernatants, obtained after centrifugation at 20,000 x g for 30 min, were subjected to acetone precipitation (final acetone concentration = 75%). The precipitates were collected by centrifugation (20,000 x g for 30 min), dissolved in 1M acetic acid, and lyophilized. The lyophilizate (920 mg) was dissolved in 20 ml of 1M acetic acid and divided into 4 portions. Each portion was subjected to gel filtration on a Sephadex G-75 fine column (1.8 x 137 cm, Pharmacia). An aliquot of each fraction was submitted to RIAs for BNP and ANP. Pooled fractions #53-56 exhibiting ir-BNP were lyophilized, and then subjected to immunoaffinity chromatography on an anti-BNP IgG-AFFI-GEL column (see below). The peptide fraction adsorbed on the immunoaffinity column was purified by successive reverse phase HPLCs first on a μ-Bondasphere C-18 column (3.9 x 150 mm, 300A, Waters), and then on a 219TP54 diphenyl column (4.6 x 250 mm, Vydac), with a linear gradient elution of CH_3CN in 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. The column effluents of HPLC were monitored by measuring absorbance at 210 and 280 nm. Aliquots of all the fractions from immunoaffinity chromatography and reverse phase HPLCs were submitted to RIA for BNP.

RIAs for BNP and ANP: Details on RIA for BNP-26 have been reported in a separate paper (3). Antiserum #158-4 mainly recognizes the ring portion of BNP-26 flanked by a disulfide linkage. When the antiserum was used at a final dilution of 1:360,000, peptides were measurable in a range of 1-100 fmol/tube, and α-ANP showed 0.02% crossreactivity. RIA for α-ANP was performed as reported previously (5). This RIA system had less then 0.003% crossreactivity with BNP-26 and BNP-32.

Immunoaffinity chromatography: Anti-BNP immunoaffinity column was prepared as reported for the isolation of BNP-32 (2). In brief, IgG fraction of antiserum #158-4, prepared with Protein A-Sepharose CL-4B (Pharmacia), was coupled with AFFI-GEL 10 (Bio-Rad). Samples dissolved in 0.1M sodium phosphate buffer (pH 7.4) were loaded onto the immunoaffinity column (total bed volume: 2 ml). After washing the column with the same buffer, the adsorbed materials were eluted with 1M acetic acid containing 10% CH_3CN.

Sequence analyses: Amino acid sequence analyses were performed by stepwise Edman degradation, using a gas-phase sequencer equipped with a reverse phase HPLC system to identify the resulting phenylthiohydantoin (PTH)-amino acids (Model 470A/120A, Applied Biosystems). The purified porcine γ-BNP and its chymotryptic and tryptic peptides, prepared as described below, were each submitted to the sequencer. Chymotryptic digestion of the purified γ-BNP (ca. 150 pmol) was performed with 200 ng of the enzyme (Worthington) in 50 μl of 50mM Hepes buffer containing 10mM $CaCl_2$ at 37°C for 2.5 hr. The purified γ-BNP (ca. 200 pmol) was also digested with TPCK-trypsin (200 ng, Cooper Biomed.) in 50 μl of the same buffer for 2 hr at 37°C. Chymotryptic and trpyic digests thus obtained were each subjected to reverse phase HPLC on a μ-Bondasphere C-18 column (3.9 x 150 mm) with a linear gradient elution of CH_3CN in 0.1% TFA.

RESULTS AND DISCUSSION

Heat-treated acid extracts of porcine cardiac atrium were first subjected to acetone precipitation. More than 80% of ir-BNP was recovered in the precipitates, which were then separated by Sephadex G-75 gel filtration. As shown in Fig. 1, ir-BNP was eluted as a single peak maximally at fractions #54-55. Immunoreactive ANP also emerged as a single peak maximally at fraction #54 which was later identified as the known γ-ANP by further purification (data not shown). Although the elution positions of the main peaks of ir-BNP and ir-ANP were retarded in comparison with our previous data

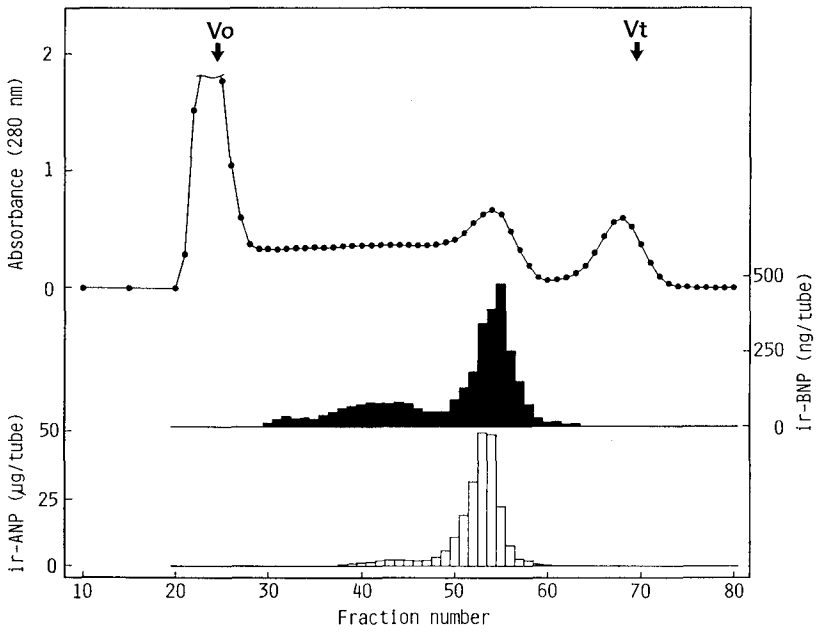

Figure 1. Sephadex G-75 gel filtration of the protein fraction prepared from acid extracts of porcine cardiac atrium.
Sample: 75% acetone precipitates of 1M acetic acid extracts of porcine atrium (25 g wet wt equivalent). Solvent: 1M acetic acid.
Column: Sephadex G-75 fine (1.8 x 137 cm, Pharmacia).
Fraction size: 5 ml/tube. Flow rate: 8 ml/hr.
BNP and ANP immunoreactivities are shown by black and white columns, respectively.

(4), ir-BNP was found to have a MW comparable to γ-ANP. After lyophilization, fractions #53-56 (total dry weight, ca. 60 mg) were subjected to anti-BNP IgG immunoaffinity chromatography. However, more than 50% of the total ir-BNP remained insoluble in the precipitates before loading onto the column. Therefore, ir-BNP in the precipitates was solubilized with Triton X-100 using an ultrasonicator, and then loaded separately onto the immunoaffinity column. After this step, a low concentration of Triton X-100 was added to solubilize high MW ir-BNP before applying samples to chromatography. The adsorbed peptides on the affinity column were eluted with 1M acetic acid containing 10% CH_3CN, pooled, and then separated by reverse phase HPLC on a μ-Bondasphere C-18 column. As shown in Fig. 2a, the peak eluted at 53 min exhibited high BNP immunoreactivity but no ANP immunoreactivity; no other peak showed significant immunoreactivity of either BNP or ANP. The ir-BNP that eluted at 53 min was finally purified by reverse phase HPLC on a Vydac diphenyl column (Fig. 2b). Based upon the peak height on final reverse phase HPLC as well as an initial yield of PTH-amino acid in the sequence analysis, the total isolation yield of the purified peptide was estimated to be about 600 pmol (7.5 μg), starting from 100 g of porcine cardiac atrium.

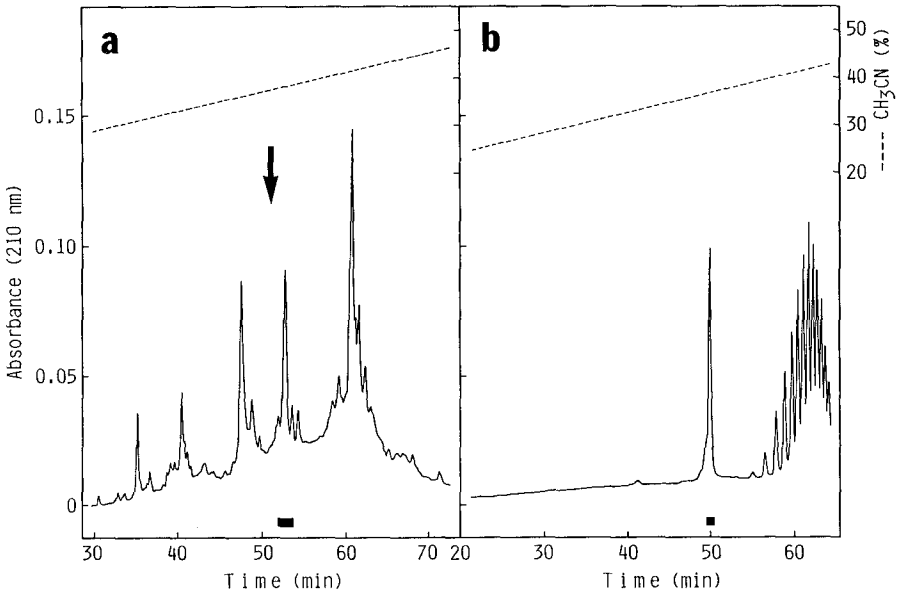

Figure 2. Successive purification of γ-BNP by reverse phase HPLC.
Sample: a) Anti-BNP immunoaffinity chromatography purified fraction obtained from fractions #53-55 in Fig. 1.
b) BNP immunoreactive fraction shown with a black bar in Fig. 2a.
Column: a) μ-Bondasphere C-18 (3.9 x 150 mm, 300A, Waters).
b) 219TP54 diphenyl (4.6 x 250 mm, Vydac).
Solvent: H_2O:CH_3CN:10% TFA = (A) 90:10:1 (v/v), (B) 40:60:1 (v/v).
Linear gradient elution from (A):(B)=80:20 to (A):(B)=0:100 for 96 min.
Flow rate: 1 ml/min. Temperature: ambient.
Black bars indicate BNP immunoreactive fractions. The arrow in Fig. 2a indicates the elution position of porcine γ-ANP. The consecutive peaks eluted after 55 min in Fig. 2b are derived from Triton X-100.

In the N-terminal sequence analysis of the purified peptide (ca. 150 pmol), PTH-amino acid liberated at each cycle of Edman degradation was successfully identified up to the 31st step from the N-terminus (Fig. 4). As a minor component, the purified peptide still contained about 25-30% of N-terminally three amino acid deleted form of the high MW BNP. However, this minor component did not disturb sequence analysis. Further structural information was provided by sequencing two series of fragmented peptides prepared by chymotryptic and tryptic digestion. Chymotryptic digestion of the purified high MW BNP yielded more than 20 peptide peaks on reverse phase HPLC, as shown in Fig. 3a. Among them, 13 peptide peaks (C1-C13) were each submitted to sequence analysis. Although peak C6 contained two fragmented peptides (C6 and C6'), the sequences of both peptides were able to be determined, since the N-terminal 31 residue sequence of high MW BNP contained the sequence of one of the C6 peptides. Peak C9 was composed of two peptide chains which were linked by a disulfide linkage and afforded two equimolar PTH-amino acids at each cycle of Edman degradation. Sequence analyses of

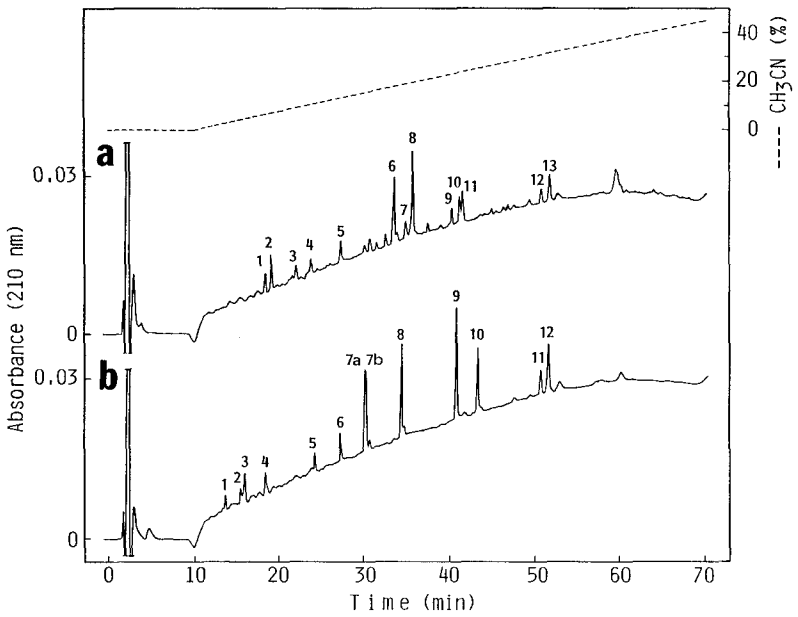

Figure 3. Reverse phase HPLC of chymotryptic and tryptic digests of γ-BNP.
Sample: a) Chymotryptic digests of purified γ-BNP (ca. 2.0 μg).
b) Tryptic digests of purified γ-BNP (ca. 2.5 μg).
Column: μ-Bondasphere C-18 (3.9 x 150 mm, 300A, Waters).
Solvent: H_2O:CH_3CN:10% TFA = (A) 100:0:1 (v/v), (B) 40:60:1 (v/v).
Linear gradient elution from (A) to (B) for 80 min.
Flow rate: 1 ml/min. Temperature: ambient.

tryptic peptides were performed in order to overlap chymotryptic peptide sequences. Tryptic digestion of the purified high MW BNP yielded about 20 peptide peaks on reverse phase HPLC, and peak T7 was further separated into two peptides (T7a and T7b) by another HPLC system (data not shown). A total of 13 peptides (T1-T12) were analyzed for their sequences (Fig. 3b). In all tryptic peptides, amino acid sequences were determined up to their C-termini. Peak T9 consisted of two peptides cross-linked by a disulfide linkage. Based on the sequences of chymotryptic and tryptic peptides along with the N-terminal 31 amino acid sequence and BNP-32 sequence, the complete 106 amino acid sequence of porcine atrial high MW BNP was finally established (Fig. 4). The structures corresponding to low MW BNPs (BNP-26 and BNP-32) were found at the C-terminus of this high MW peptide, in the same position that α-ANP was present in γ-ANP.

Concurrent with the present study, we have cloned and analyzed a cDNA sequence encoding a precursor of porcine BNP (to be reported elsewhere). The present sequence analysis data for the high MW BNP was found to be exactly identical to the sequence [26-131] in the prepro-form of porcine BNP deduced from cDNA analysis. Judging from structural features, the 25 residue peptide at the N-terminus of the prepro-BNP was thought to be a signal peptide and the

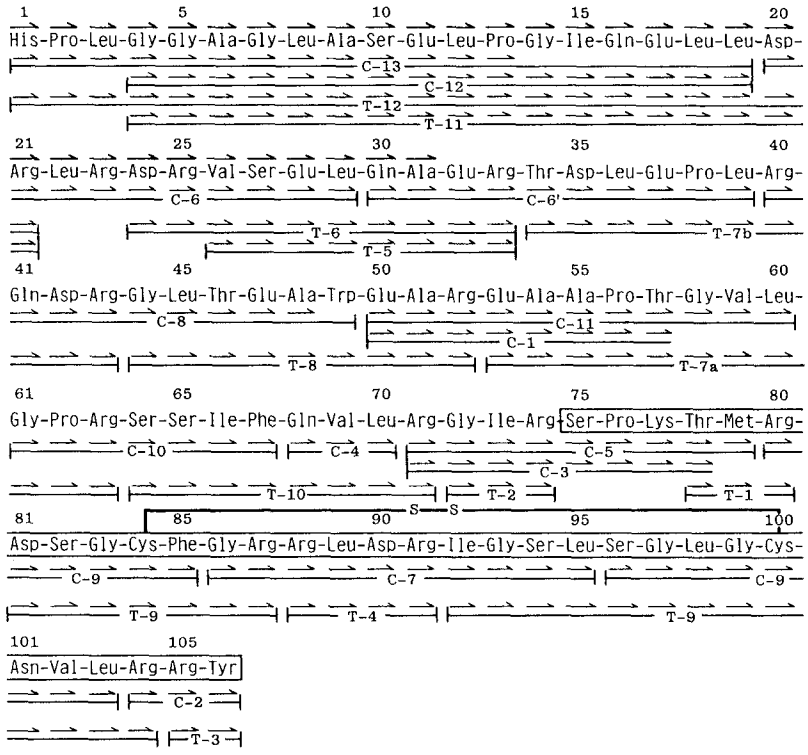

Figure 4. Complete amino acid sequence of porcine γ-BNP. Amino acid sequence analyses were performed directly with γ-BNP as well as with its chymotryptic (designated C) and tryptic peptides (designated T). C1-C12 and T1-T13 correspond to the peptides indicated in Figs. 3a and 3b. The arrows indicate amino acid residues identified by stepwise Edman degradation. The sequence boxed with a solid line is BNP-32.

porcine high MW BNP identified in the present study was found to correspond to pro-BNP. Just as in the case of the γ-form in the ANP family, this pro-BNP was found to be a predominant form of BNP in heart tissue, representing the γ-form in the BNP family. Therefore, we have designated this high MW peptide as "porcine γ-BNP".

When the amino acid sequence of porcine γ-BNP is compared with that of porcine γ-ANP, significant homology is observed only in the region corresponding to BNP-32 (6-8). Comparing with γ-ANP, γ-BNP is 20 residues shorter in length and has only 31.6% identical residues when aligned to show maximum homology. Taking into account the fact that more than 73.8% of all total residues are identical within the mammalian ANP family (9), BNP and ANP are thought to have diverged in the early stage of evolution.

Figure 5 summarizes molecular forms and biosynthetic pathways of BNP and ANP in porcine heart and brain. As reported in our recent paper (4), more than 70% of ir-BNP in the acid extract of cardiac atrium exists as a high MW form. In the present study, the high MW ir-BNP has further been identified as

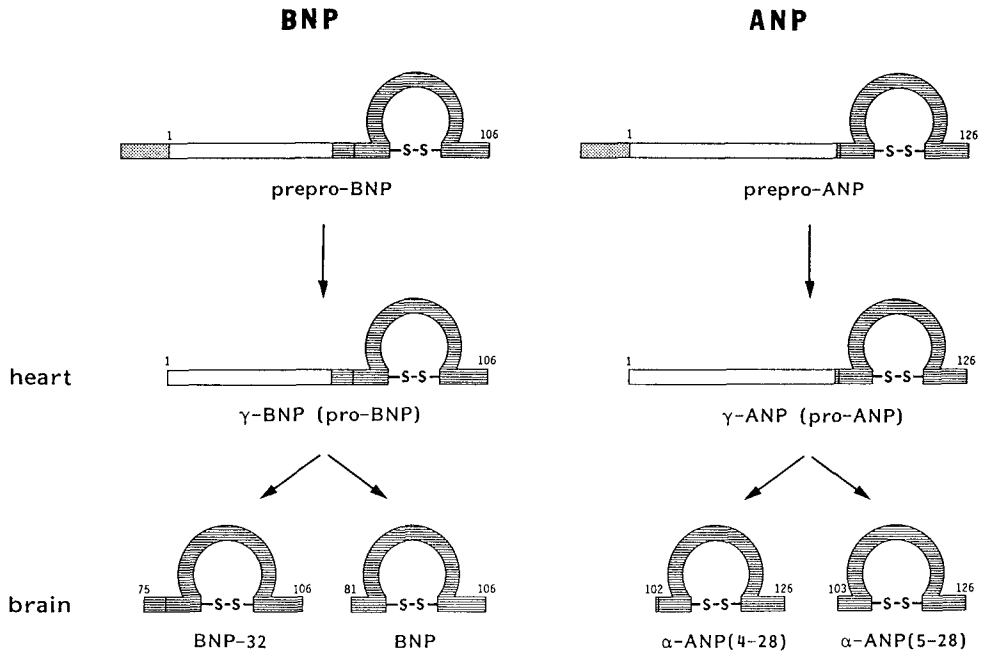

Figure 5. Schematic representation of molecular forms and biosynthetic pathways for BNP and ANP in porcine heart and brain.

γ-BNP (pro-BNP), which is generated from a 131-residue precursor by removal of a 25-residue signal peptide. These results indicate that γ-BNP is the major storage form in porcine cardiocyte in the same manner as that for mammalian ANPs and that no proteolytic processing takes place after removal of the signal peptide in the heart (5,10,11). In contrast, we have identified BNP-26 and BNP-32 of low MW as being the major endogenous forms of BNP in porcine brain (1-3), indicating that further processing takes place to generate these low MW BNPs as was observed in the ANP family in brain, where α-ANP[4-28] and α-ANP[5-28] were shown to be predominant forms (12). These facts demonstrate that both BNP and ANP have similar patterns in the post-translational processing and biosynthetic systems in heart and in brain.

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