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Brain natriuretic peptide-like immunoreactivity is present in human plasma

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A highly specific and sensitive radioimmunoassay (RIA) for a novel porcine brain natriuretic peptide (BNP) has been established to elucidate whether BNP-like immunoreactivity (LI) is present in human plasma. The antibody used was specific for BNP without any crossreactivities with known human atrial natriuretic peptides (hANP). After extraction of human plasma with Sep-Pak cartridge, this assay allowed for detection of BNP-LI as low as 0.1 fmol/tube. In 12 healthy subjects, the mean concentrations of plasma BNP-LI were 1.5 fmol/ml. Reverse-phase HPLC coupled with BNP RIA revealed that the single major component with BNP-LI, corresponding to porcine BNP(1–26), was apparently distinct from that of α-hANP. These data indicate that a small molecular mass form with BNP-LI circulates in human blood.

Natriuretic peptide; Plasma; RIA; HPLC, reverse-phase; (Brain)

1. INTRODUCTION

Brain natriuretic peptide (BNP) has recently been isolated and sequenced from the extract of porcine brain [1]. Porcine (p) BNP consists of 26 amino acid residues with a remarkable homology to, but definitely distinct from the sequence of atrial natriuretic peptide (ANP). pBNP elicits a pharmacological spectrum of diuretic/natriuretic and hypotensive effects very similar to that of ANP [1]. pBNP appears to interact with the same ANP receptor sites in rat vascular smooth muscle cells [2] and bovine adrenal glomerulosa cells [3]. Subsequently, a large molecular mass BNP, designated as γ-BNP [4], has been identified in the porcine cardiac atrium. γ-BNP comprises 106 amino acid residues carrying BNP at its C-terminus, and serves as a possible precursor for BNP. It has recently been reported that a small molecular mass BNP is secreted into the perfusate from the isolated porcine heart [5]. These results suggest that BNP is also synthesized by the heart and is secreted into the circulation in a similar fashion to ANP. However, there has been no information available as to whether BNP circulates in human blood. Therefore, the present study was designed to establish a highly sensitive and specific radioimmunoassay (RIA) for BNP to measure concentrations of BNP-like immunoreactivity (LI) in normal human plasma, and to characterize the circulating form by reverse-phase HPLC.

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2. MATERIALS AND METHODS

2.1. *Production of antibody for pBNP*

Synthetic pBNP (Peptide Institute, Inc., Osaka, Japan) was conjugated to bovine thyroglobulin (Sigma Chemical, St. Louis, MO) using carbodiimide as a coupling agent. The conjugate emulsified with complete Freund's adjuvant (Difco Labs, Detroit, MI) was injected intradermally at multiple sites on the back of Japanese white rabbits over a period of 12 weeks at 3–14-day intervals. After repeated immunization, an antiserum suitable for RIA was obtained.

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2.2. *Iodination of pBNP*

pBNP was radioiodinated by the lactoperoxidase method. pBNP (12 μg) in 20 μl distilled water, 40 μl of 10 mM phosphate buffer, pH 5.6, 5 μl Na[^{125}I] (1 mCi, New England Nuclear, Boston, MA), 0.624 μg lactoperoxidase (Sigma Chemical, St. Louis, MO) in 5 μl phosphate buffer, and 10 μl of 1.8 mM H_2O_2 were added sequentially. The reaction was allowed to proceed for 30 min at room temperature, and terminated by the addition of 50 μl of 50 mM mercaptoethanol. Labeled pBNP was purified by using reverse-phase HPLC. The specific activity of [^{125}I]BNP ranged from 510 to 600 μCi/μg.

2.3. *RIA of BNP*

RIA for BNP was performed essentially as reported for ANP RIA [6]. In brief, 0.1 ml sample or standard, 0.1 ml assay buffer and 0.1 ml anti-BNP-serum (final dilution, 1:420000) were incubated at 4°C for 24 h, followed by the addition of 0.05 ml [^{125}I]BNP (approx. 10000 cpm) and further incubation at 4°C for 24 h. The bound ligands were separated from free ligands by the double antibody method. The standard curve was constructed using B/B_0 vs pBNP standard. The amount of BNP in the unknown samples was extrapolated from the standard curve linearized by a logit-log transformation.

2.4. *Plasma extraction*

2 ml aliquot of human plasma acidified with trifluoroacetic acid (TFA) was applied to a Sep-Pak C_{18} cartridge (Waters, Milford, MA) prewashed sequentially with methanol, distilled water and 0.09% TFA. The material(s) absorbed to the cartridge was eluted with 2 ml of 60% acetonitrile/0.09% TFA and evaporated. The dried residues were reconstituted in the assay buffer and subjected to RIA for BNP and α-ANP [6]. For chromatographic analysis of plasma BNP-LI, an aliquot of human plasma extract was subjected to reverse-phase HPLC.

2.5. *Reverse-phase HPLC*

Reverse-phase HPLC was performed using an octadecyl-silica column (0.45 × 25 cm, Jasco, Tokyo, Japan) eluted with a linear gradient of acetonitrile from 15% to 60% in 0.09% TFA for 1 h with a flow rate of 1 ml/min. 1-ml fractions were collected and assayed for BNP-LI and α-hANP-LI.

3. RESULTS

As shown in fig.1, the minimum detectable quantity of pBNP was 0.1 fmol/tube (99% confidence), and the 50% binding intercept was 1.5 fmol/tube. The intra- and inter-assay coefficients of variation (n = 5) were 6 and 10%, respectively. The antibody crossreacted less than 0.001% with α-hANP, β-hANP, γ-hANP and rat α-ANP on a molar basis (fig.2); it had no crossreactivities with angiotensin II or arginine-vasopressin. The dilution curves generated by human plasma extracts were parallel to that of standard pBNP (fig.1). The recovery of unlabeled pBNP added to human plasma was 75.9 ± 3.0% (n = 3) throughout the extraction procedure. In 12 normal subjects (7 males and 5 females, aged 28.3 ± 9.6 year-old), the mean concentrations of BNP-LI and α-hANP-LI were 1.5 ± 0.4 fmol/ml and 5.8 ± 1.7 fmol/ml, respectively.

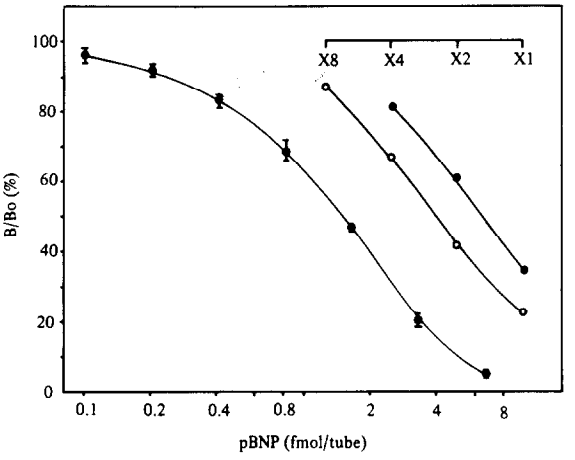

Fig.1. Radioimmunoassay for BNP. Standard curve for pBNP(⊕: mean ± SD, n = 4) and dilution curves of normal human plasma extracts (○,●) are shown. Serial dilutions of the human plasma extracts are denoted in the upper panel.

Elution profiles of BNP and α-hANP-LI in human plasma extract by reverse-phase HPLC are shown in fig.3. A single major component with BNP-LI coeluting with authentic pBNP(1-26) was observed (fig.3, upper panel), while one major component with α-hANP-LI corresponding to the position of authentic α-hANP(1-28) was eluted earlier than pBNP (fig.3, lower panel).

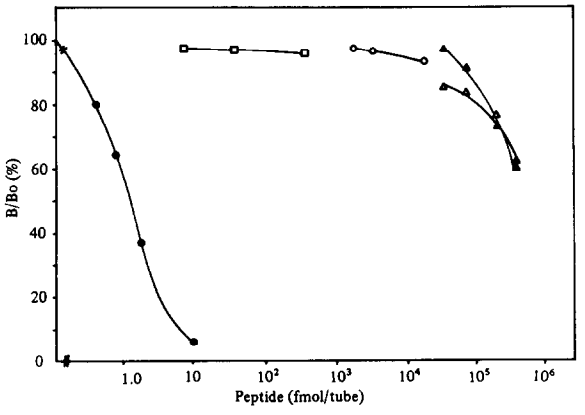

Fig.2. Crossreactivities of ANP and related peptides in BNP RIA. Serial dilution curves of pBNP(1-26) (●), α-hANP (△), β-hANP (○), γ-hANP (□), and α-rat ANP (▲) are shown.

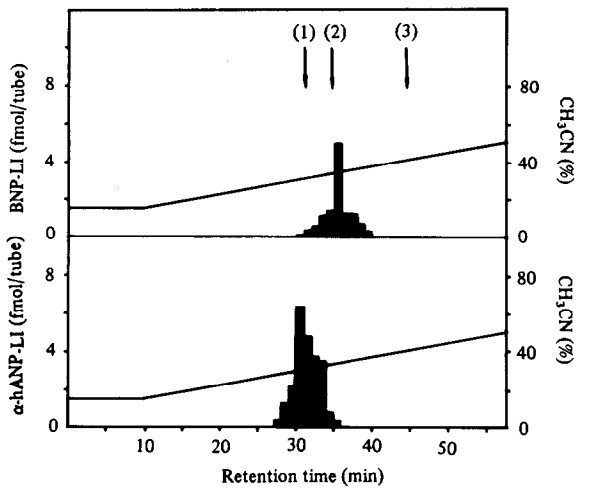

Fig.3. Reverse-phase HPLC profiles of BNP- and α-hANP-LI in human plasma extract. An aliquot of human plasma extract (3.5 ml) was subjected to a reverse-phase HPLC. Concentrations of BNP-LI (upper panel) and α-hANP-LI (lower panel) are shown by the solid columns. Arrows indicate the elution positions of α-hANP(1), pBNP(2) and γ-hANP(3), respectively. A linear gradient of acetonitrile from 15 to 60% is denoted by solid lines.

4. DISCUSSION

By using sensitive and specific RIA for BNP with a minimum detectable dose of 0.1 fmol of pBNP, we were able to measure BNP-LI in normal human plasma. The antibody used in the present RIA was specific for pBNP without any crossreactivities with structurally similar hANP family, including α-hANP, β-hANP and γ-hANP. pBNP shows a remarkable sequence homology to ANP (70%), but has 7 amino acid replacements and one insertion compared to α-hANP(4–28) [1]. Therefore, it is suggested that the principal antigenic determinant of the antibody is directed toward the residues not shared by the hANP molecule.

Dilution curves of human plasma extracts appear to be parallel to that of standard pBNP, suggesting that the material(s) immunologically indistinguishable from pBNP is present in human plasma. We have also observed significant amounts of BNP-LI in the human atrial tissues, but not in the ventricular tissues (unpublished). Although the complete amino acid structure of human BNP is not determined yet, our data strongly suggest that BNP structurally very similar, if not identical, to pBNP is also present in human plasma and heart.

Plasma concentrations of BNP-LI in normal subjects (1.5 fmol/ml) are comparable to those of porcine plasma (1.4 fmol/ml) [3], but about 4-fold lower than those of α-hANP-LI.

Reverse-phase HPLC coupled with RIAs for BNP and α-hANP revealed that BNP-LI in human plasma consisted of only one major component with identical retention time to that of authentic pBNP(1–26), apparently distinct from that of α-hANP-LI, suggesting that BNP is more hydrophilic in nature than α-hANP. These data are also consistent with those of a recent report [5] in which the major BNP-LI in the perfusate of the isolated porcine atrium is a small molecular mass component corresponding to authentic pBNP. Taken together, it is suggested that the main secretory and circulatory form of BNP in pigs and man is a small molecular mass component in analogy to α-hANP. It remains to be determined whether the circulating BNP-LI in human plasma derives mainly from the human heart and whether BNP and ANP are co-secreted under physiological and pathological conditions.

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