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MOLECULAR FORMS OF ATRIAL NATRIURETIC POLYPEPTIDES IN MAMMALIAN TISSUES AND PLASMA

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SUMMARY: A highly sensitive radioimmunoassay detecting for all the atrial natriuretic polypeptides isolated so far from human and rat(hANPs and rANPs) has been established by using an antiserum raised agaisnt α-hANP, since the antiserum recognizes the subsequence flanked by two cysteine residues(positions 7 and 23) in α-hANP and crossreacts with human as well as rat ANPs. By using the radioimmunoassay combined with gel chromatography or high performance liquid chromatography, it was revealed that ANP immunoreactivity in human atria is composed of α-(28 residues), β-(56 residues:α-hANP dimer) and γ-hANP(126 residues) in various ratios. In rat, porcine and bovine atrial tissues, however, γ-rANP(126 residues) was found to be a major component. In contrast with atrial ANP, plasma ANP was found to be predominantly α-form in rat.

Atrial natriuretic polypeptides (ANP), eliciting potent natriuretic-diuretic activity, have been isolated from human(1-3) and rat(4-11) atrial tissues and implicated in the control of fluid volume and vascular function. From human atria, three distinct forms(α;28, β;56, γ;126 amino acid residues) have been isolated(1-3). α-hANP has been first identified as a 28-residue peptide containing a disulfide linkage(1) and β-hANP is known to be an antiparallel dimeric form of α-hANP(2). Furthermore, γ-hANP of 126 residues has been revealed to be an N-terminally extended peptide of α-hANP, which is the largest form processed out of a 151-residue precursor(2,3). In rat atria, a 28-residue peptide, designated α-rANP(4) or cardionatrin(5) has been identified as the peptide identical with α-hANP having a single amino acid replacement at position 12 of Ile for Met. In addition, γ-rANP of 126 residues, which is highly homologous to γ-hANP, has also been identified as the largest form of rANPs(11). However, a number of N-terminally extended or deleted forms of α-rANP, varying in the chain length, have been isolated by various research groups, though in very low yield and named variously (6-10). All of them have been found to be truncated peptides of γ-rANP. Identification of cDNAs encoding the human (3) and rat (11-14) ANP precursors revealed that sequences of all the atrial peptides isolated to date are contained within the precursor molecules, though their processing features remain uncertain. It is essential to solve the question as to which molecular forms of ANPs are present in and secreted from

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the cardiocytes and circulating in the blood. In this context, we have established a highly sensitive radioimmunoassay(RIA) for ANPs to directly measure the ANP content, as described in the present paper. By using the RIA, combined with chromatographic separation, we identified molecular forms of ANP present in heart and in blood of mammals.

MATERIALS AND METHODS

Materials: α-hANP and α-rANP were synthesized in our laboratory(1,4). β-hANP, γ-hANP, and γ-rANP were each purified from human and rat atrial extracts as described(2,11). α-hANP(1-27), (7-28), (13-28), and (18-28) were kindly donated from Dr. Minamitake, Suntory Institute of Biomedical Research, Osaka, Japan. α-hANP(1-26) was prepared by carboxypeptidase B digestion of α-hANP(1-27). Reductive S-carboxymethylation (RCM) of α-hANP was performed by the described method(1). Bovine serum albumin(BSA: Sigma, fraction V) for the RIA was pretreated with 5mM N-ethylmaleimide for 24 hr at room temperature, and twice recrystallized.

Preparation of antiserum for α-hANP: α-hANP was conjugated with bovine thyroglobulin(Sigma) by the carbodiimide method as decribed previously(16). Antigenic conjugate solution prepared as above was emulsified with an equal volume of Freund's complete adjuvant, and used for immunizing New Zealand white rabbits by subcutaneous injection at multiple sites in the interscapulo-vertebral region.

Radioiodination: α-hANP(10 μg) in 25 μl of 0.4M sodium acetate buffer (pH 5.6) was introduced into a tube followed by the addition of $Na^{125}I$ (1mCi/10μl, Amersham). Lactoperoxidase (Boehringer Mannheim: 20ng / 10μl of 0.1M sodium acetate, pH 5.6) and H_2O_2 (100ng / 5μl of water) were added into the reaction tube. After left for 10 min at 30°C, H_2O_2 (100ng / 5μl of water) was added into the tube, which was left for another 10 min at 30°C. Immediately after reaction, the mixture was submitted to reverse phase HPLC, where two major radioactive peaks were obtained. After removal of the first peak (monoiodinated α-hANP sulfoxide), monoiodinated α-hANP eluted in the second peak was purified and used as a tracer, which was stored at -80°C until use.

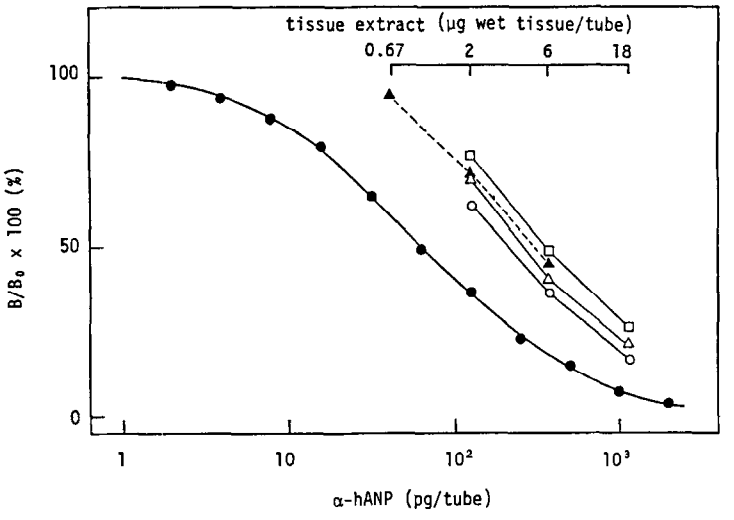
Procedures for RIA: The incubation buffer for RIA was 0.05M sodium phosphate buffer (pH 7.4), containing 1% BSA, 0.1% Triton X-100, 0.08M NaCl, 0.025M EDTA·2Na, 0.05% NaN_3, and Trasylol 500 KIU/ml. The standard α-hANP or the unknown sample (100 μl) was incubated with anti-α-hANP antiserum diluent (500 μl) for 12 hrs. Then the tracer solution (18,000-20,000 cpm in 200 μl) was added. A glass tube (10 x 75mm) was used for assay. All assay procedures were performed at 4°C. After the incubation for 36 hrs, anti-rabbit IgG goat serum diluent (200 μl) was added. After kept standing for 40 hrs, the tubes were centrifuged at 2,000g x 30 min at 4°C and radioactivity of the precipitate was measured in Aloka ARC-600 gamma counter.

Preparation of tissue and plasma samples: i) Tissue sample: Human atrial tissue was resected at autopsy within 4 hrs post mortem. Rat atrial tissue was taken immediately after decapitation. Porcine and bovine atrial tissues were obtained at slaughter house. The tissues were homogenized with a polytron mixer for 60 sec in 20 volume (V/W) of 0.1M AcOH containing 1% Triton X-100 and immediately boiled for 5 min to inactivate proteases. After chilling, the homogenate was centrifuged at 25,000g x 20 min. The supernatant was pooled and stored at -20°C before use. ii) Plasma samples: Plasma samples were taken with EDTA·2Na (1 mg/ml) and Trasylol (500 KIU/ml), and applied on a Sep-Pak C-18 cartridge(Waters), which was preequilibrated with saline. After washing the column with saline, the adsorbed material was eluted with 60% acetonitrile in 0.1% trifluoroacetic acid(TFA). The eluate was evaporated and subjected to reverse phase HPLC.

RESULTS AND DISCUSSION

Characterization of the antiserum: The antiserum (#125-8) was proved to be usable at a final dilution of 1:400,000 for RIA of α-hANP, utilizing ^{125}I-labeled ligand. As seen in Fig.1, half maximum inhibition by α-hANP was observed at 60 pg/tube and α-hANP was detectable as low as 10 pg/tube. The specificity of the antiserum was evaluated from its crossreactivity with several ANP-related peptides(Fig.1), indicating that the antiserum mainly directs to the subsequence flanked by two cysteine residues(positions 7 and 23) in α-hANP molecule. The antiserum shows 40% crossreactivity with α-rANP, which is a 28-amino acid peptide identical to α-hANP, having an only single residue replacement (Met^{12}← Ile^{12}). In addition, γ-rANP(126 residues), which is an N-terminally extended form of α-rANP, and all other rANPs so far isolated also crossreact appreciably with the antiserum(40%). Thus, hANPs and rANPs were found to be precisely measured by this RIA system. However, it should be mentioned that special care must be taken in the assay: i) ^{125}I-hANP must be purified immediately after iodination by reverse phase HPLC and stored -80 °C before use. ii) BSA used for RIA buffer must be treated with N-ethylmaleimide before use. Otherwise, free thiol group in BSA may participate in opening and rejoining of disulfide bond of ANPs to form BSA-ANP conjugates, which interfere the assay. iii) The C-terminal Tyr carrying ^{125}I is so susceptible to proteolytic cleavage, that addition of Trasylol in the assay buffer is essential for obtaining reproducible results.

ANP in atrial tissue: The present procedure for tissue extraction is quite efficient to prevent nonspecific degradation of ANP by intrinsic proteases. Dilutions of the acid extracts from human, rat, bovine and porcine atrial tissues yielded competition curves which were parallel to the standard curve of α-hANP(Fig.1). Thus, the present RIA can precisely detect all the known forms of both human and rat ANPs so far identified. Furthermore, bovine and porcine(15) ANPs, which remain to be identified, can also be detected by this RIA. Fig.2a shows a typical gel-filtration pattern on Sephadex G-75 of rat atrial extract, monitored by the RIA, where only one major immunoreactive peak, with more than 90% of the total immunoreactivity, was observed at the position corresponding to γ-rANP (Mr 13,000). Though gel-filtration data similar to or different from ours, possibly due to differences in procedures for tissue extraction, have been reported, definite identification of the immunoreactive species in atrial tissue has not been done as yet(17-19). For unambiguous identification, we have performed further chromatographic comparison with authentic γ-rANP on reverse phase HPLC. As shown in Fig.2d, nearly all of the ANP-immunoreactivity of rat atria comigrated exactly with γ-rANP. Thus, γ-rANP was a predominant form of rANP in rat atrial tissue.



The crossreactivity of anti α-hANP antiserum (#125-8)

peptides	(%)
α-hANP	100
β-hANP	100
γ-hANP	100
α-hANP[1-26]	100
α-hANP[1-27]	100
RCM-α-hANP	55
α-hANP[7-28]	100
α-hANP[13-28]	8
α-hANP[18-28]	< 1
α-hANP sulphoxide	100
α-rANP	40
γ-rANP	40

Fig.1 Standard curve of radioimmunoassay for α-hANP(●——● ^{125}I-α-hANP) and crossreactivity of the antiserum(# 125-8). Inhibition of binding to the antiserum by serial dilution of each extract from human atria (○——○), rat atria (▲——▲), porcine atria (□——□) and bovine atria (△——△), respectively. The dilution curves are parallel with that of standard α-hANP.

Although a variety of truncated peptides of γ-rANP with low or intermediate molecular size (Mr 3,000-5,000) has been isolated from rat atrial extracts(4-9), no significant quantities of immunoreactive species corresponding to these peptides were observed in the present experiments, despite the fact that the antiserum employed recognizes all of these peptides. Accordingly, it is concluded that γ-rANP is the only major component present in rat atrial tissues. Considering past observations that isolation yield of each smaller peptide was very low, representing not more than 1% of that of γ-rANP, the present data indicate that the low- and intermediate-sized peptides may be not endogenous ANPs, but secondary products derived from γ-rANP by non-specific proteolysis during isolation. As shown in Fig.2b,c,e,f, essentially similar molecular distributions of ANP-immunoreactivity were also observed in atrial tissues of porcine and bovine species.

However, the molecular patterns in human atria showed some complexity, representing three distinct types(I,II,III), with 12 autopsied human hearts thus far tested. Figure 3a shows a gel-filtration chromatography on Sephadex G-75 of the extract of human autopsied atrial tissue(type I). There were observed two major immunoreactive peaks of hANP, of which the first smaller peak eluted at a molecular weight of γ-hANP and the larger peak at that of β-hANP. In Fig.3b, reverse phase HPLC of the same extract clearly indicates that a major immunoreactivity peak is identical with the authentic β-hANP, while a minor peak identical with the authentic γ-hANP. Further confirmation of β-hANP was made after conversion to RCM-α-hANP, which was identified by HPLC

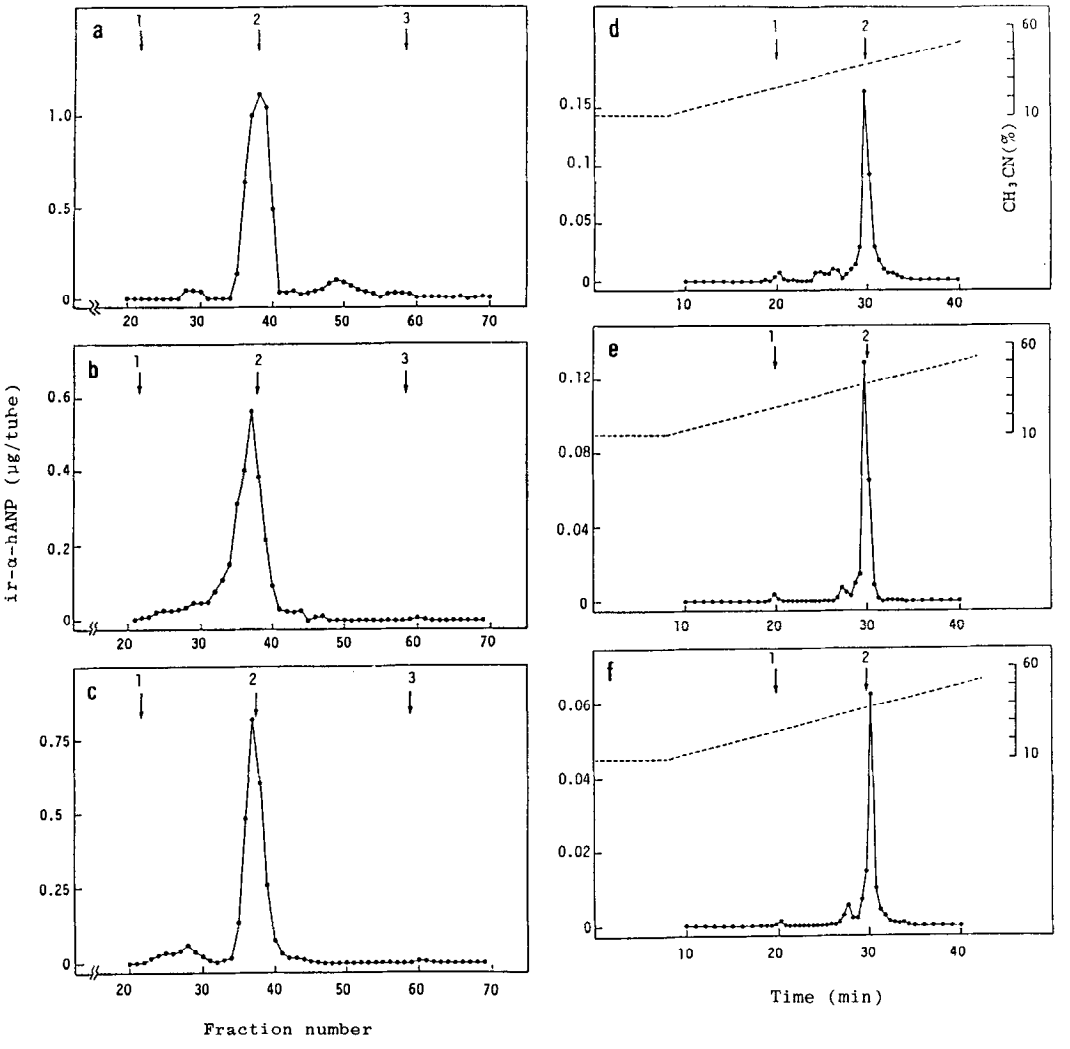


Fig.2 (a)(b)(c): Gel-filtration of atrial extract (200mg eq.): (a) rat, (b) porcine and (c) bovine. Column: Sephadex G-75 (1.8 x 135 cm). Eluent: 1M AcOH. Fraction size: 5 ml/tube. Arrows indicate elution positions of molecular weight, (1) void volume, (2) γ-rANP (Mr 13,000), (3) α-rANP (Mr 3,000).
(d)(e)(f): Reverse phase HPLC of atrial extract (50mg eq.): (d) rat, (e) porcine and (f) bovine. Column: TSK LS-410 ODS SIL(4.0 x 250mm, Toyosoda). Solvent system: H_2O : CH_3CN : 10% TFA = (A) 90 : 10 : 1, (B) 40 : 60 : 1 (v/v). A linear gradient from (A) to (B) for 40 min. Flow rate: 2 ml/min. Arrows indicate elution positions of (1) α-hANP and (2) γ-hANP.

comparison with authentic specimen. Thus, the human atrium of type I contains two components comprising β- and γ-hANP. However, another type of autopsied heart (Type II) shows a different pattern(Fig. 3c), indicating the presence of three components; α-, β- and γ-hANP, of which γ-hANP was found to be the major part. Type III, as shown in Fig.3d, exhibits a pattern very similar to those observed in rat, porcine and bovine tissues, where γ-ANP is present as a single major component. It should be noted that β-form of ANP has been found only in

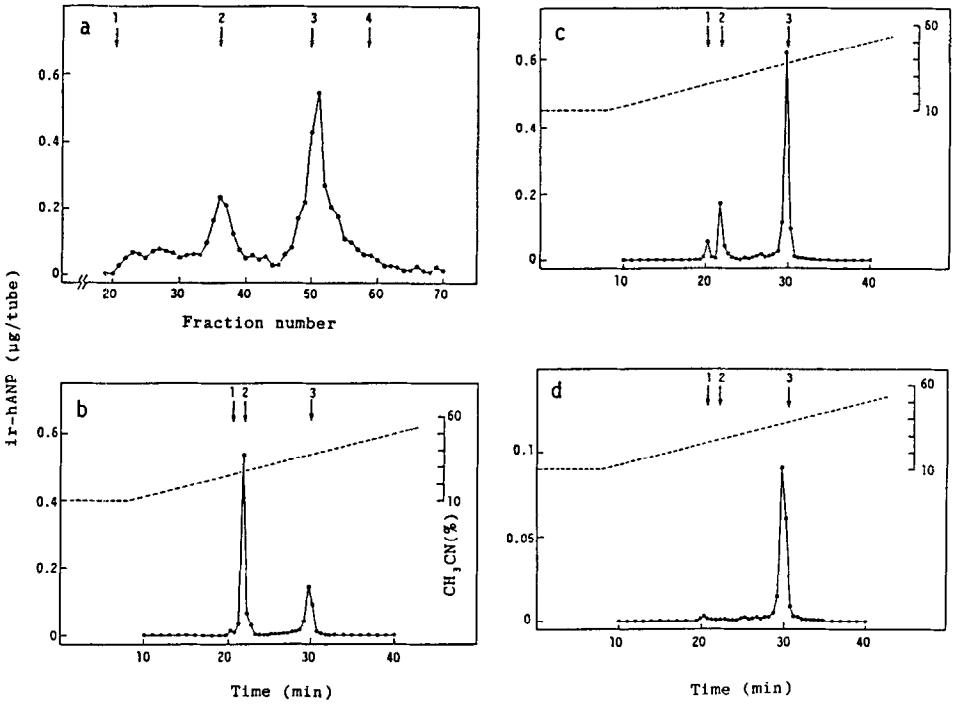


Fig.3 (a): Gel-filtration of crude extract (200mg eq.) from human atria (type I: from a 78y male). Chromatographic conditions were as in Fig.2a,b,c. Arrows indicate the elution positions of molecular weight, (1) void volume, (2) γ-hANP (Mr 13,000), (3) β-hANP (Mr 6,000), (4) α-hANP (Mr 3,000).
(b)(c)(d): Reverse phase HPLC of crude extract (50mg eq.) from human atria: (b) type I (as in Fig.3a), (c) type II (from a 63y male) and (d) type III (from a 35y male). Arrows indicate elution positions of (1) α-hANP, (2) β-hANP and (3) γ-hANP. Chromatographic conditions were as in Fig.2d,e,f.

human, but never in other mammals. Further studies are required to account for this remarkable difference in molecular distribution of ANPs between human and other mammals. Although the human atria tested were specimens autopsied from the patients without cardiovascular, renal diseases and hypertension, a possibility that these differences might reflect unknown pathological states affecting ANP content in the atria can not be ruled out. Incidentally, the content of ir-hANP widely varied from 6.11 to 47.8 ng/mg wet tissue. It should be noted that type I, where β-hANP is major component, showed a relatively higher level of ANP content in the tissue, compared with other types in which γ-hANP was predominant. In addition, among the hearts of type I, remarkable differences in molar ratio of β- vs γ-hANPs were observed ranging from 1.2/1 to 7.1/1. These data may imply that formation of β-form could be implicated with overproduction or accumulation of hANP in the cell, although the mechanisms for conversion of γ-ANP to α-ANP, and for β-formation are to be clarified.

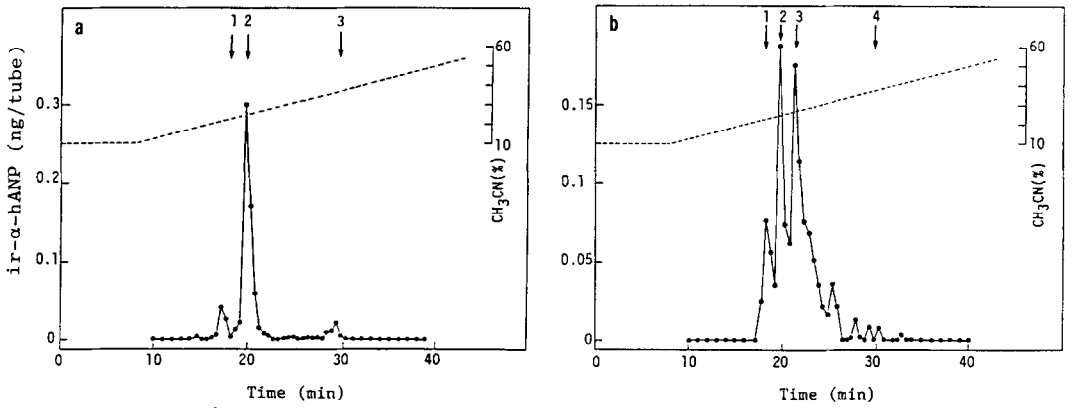


Fig.4 Reverse phase HPLC of rat (a) and human (b) plasma. The plasma samples of rat (3 ml) and human (5 ml) were pretreated with Sep-Pak C-18 as described in the text. Chromatographic conditions are the same in Fig.2d,e,f. Arrows indicate the positions of the authentic peptides: in (a): 1, α-hANP[1-26]; 2, α-rANP; 3, γ-rANP.
in (b): 1, α-hANP[1-26]; 2, α-hANP; 3, β-hANP; 4, γ-hANP.

ANP in plasma: In gel-filtration of rat plasma, we observed one major immunoreactive peak appeared in the range of lower molecular weight (Mr 3,000), along with minor peaks in the higher molecular weight region (data not shown). Fig.4a shows HPLC pattern of rat plasma, where a predominant peak possessing 90% of the total immunoreactivity emerged at the position exactly identical with the authentic rat α-ANP(28 residues), positively indicating that a major component of the ANP in the circulation is α-form.

Different from rat plasma, human plasma showed some complex feature, as observed in the atrial tissue. As shown in Fig.4b, the HPLC of human plasma indicates that the ANP immunoreactivity comprises α- and β-hANP, along with other minor components so far unidentified. Further study of ANP components to be secreted in the circulation is now in progress in our laboratory.

Incidentally, the content in rat plasma (Wistar rats, n=5) was found to be 389.3 ± 53.0 pg/ml for α-rANP eq. The content of ir-α-hANP in healthy humans was determined to be 231 ± 37.1 pg/ml, by RIA using 100 µl of plasma specimens (n=6, average age 24.8y).

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