Demonstration and characterization of α-human atrial natriuretic factor in human plasma

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This paper describes a highly specific and sensitive radioimmunoassay for α-human atrial natriuretic factor (α-hANF), the C-terminal 28-amino-acid residue portion of human prepro-ANF in human plasma. A novel extraction and prepurification procedure allowed for detection of levels of immunoreactive-α-hANF as low as 0.5 fmol/ml. In normotensive subjects, levels in the range 1–23 fmol/ml (mean = 8.9 fmol/ml) were found. Combined gel permeation and HPLC analysis demonstrated that this ir-α-hANF was comprised virtually exclusively of authentic 28-residue α-hANF. No evidence for occurrence of larger precursor forms in human plasma was acquired. A heterogenous group of hypertensive patients displayed considerably higher levels (mean = 62.2 fmol/ml), of interest in view of the hypotensive properties of ANF.

Atrial natriuretic factor *Human plasma* *Extraction* *Chromatographic characterization* *Hypertension*

1. INTRODUCTION

The atrial natriuretic factors (ANF) comprise a family of naturally occurring peptides possessing natriuretic, diuretic [1] and smooth muscle relaxant properties [2]. They were originally isolated and characterized from rat heart [3–5] and their presence in the plasma of the rat [6,7] has been established. Recently, it proved possible to demonstrate their occurrence in human heart [8], but as yet no method allowing for their reliable determination and characterization in human plasma is available. This would appear to be of particular importance since, via the systemic circulation, they may play a role in modulation of, for example, extracellular volume or electrolyte balance and may be involved in the pathophysiology of conditions such as primary or secondary hypertension. In addition, measurement of their levels in plasma would allow for the monitoring of their release and of the functional activity of ANF systems. We have, therefore, developed a method for the extraction, determination and characterization of α-hANF, the C-terminal 28-amino-acid residue portion of the human precursor, prepro-ANF [9], in human plasma and initiated studies involving the evaluation of the levels of this in various pathological states.

Abbreviations: ir, immunoreactive; RIA, radioimmunoassay; HPLC, high-performance liquid chromatography; HPGPC, high-performance gel permeation chromatography; α-hANF, α-human ANF; α-rANF, α-rat ANF; AP I,II,III, atriopeptin I,II,III

2. MATERIALS AND METHODS

2.1. *Generation of α-hANF-directed antibodies*

Human α-ANF (Nova Biochem, Switzerland) was covalently conjugated to bovine thyroglobulin employing carbodiimide as a coupling agent as in [10]. A conjugate fraction was emulsified with an equal volume of complete Freund's adjuvant and 2 ml emulsion (equivalent to 800 μg α-hANF) was injected intradermally at multiple sites on the back

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of 3 male New Zealand rabbits. At intervals of 4 weeks, animals were boosted with 200 μg ANF conjugate emulsified in Freund's incomplete adjuvant. 4 weeks following booster, serum was collected, divided and stored at −30°C.

2.2. *Preparation of ^{125}I-α-hANF*

1 mCi ^{125}I in a volume of 10 μl was added to 5 μg α-hANF in 50 μl of 0.5 M sodium phosphate buffer, pH 7.2. The reaction was initiated by the addition of 10 μl chloramine T solution (20 μg in 0.05 M sodium phosphate buffer, pH 7.4). The mixture was shaken for 30 s and the reaction stopped by 10 μl sodium metabisulfite solution (20 μg in 0.05 M sodium phosphate buffer, pH 7.4). Iodinated α-hANF was purified by gel filtration on a 1 × 30 cm column of Bio-Gel P4 (pretreated with 1% bovine serum albumin and eluted with 0.1 M acetic acid). 1-ml fractions were collected into polypropylene tubes to which 100 μl RIA buffer were added to minimize adsorbance of the peptide to the tube walls. Fractions were frozen and stored at −30°C.

2.3. *Radioimmunoassay for α -hANF*

RIA buffer (300 μl containing 0.02 M sodium phosphate, 0.15 M NaCl, 0.01% bovine serum albumin, 0.1% gelatin, 0.01% thiomerosal, 0.1% Triton X-100) were mixed with 50 μl of α-hANF standards or samples in 1.5 ml Eppendorf plastic vials. 100 μl of antiserum 'Toni II' were added to yield a final antibody dilution of 1:35000. The reaction was initiated with ^{125}I-α-hANF in 50 μl RIA buffer and allowed to incubate at 5°C for 12–16 h. Separation of bound and free hormone was achieved by addition of 500 μl of a mixture containing 1% charcoal and 0.5% bovine serum albumin in RIA buffer without addition of Triton X-100. The suspension was left for 10 min, then centrifuged for 3 min. 800 μl supernatant was counted in a γ-counter.

2.4. *Collection of plasma samples*

Peripheral blood was drawn into pre-cooled 20 ml syringes containing 500 KIU/ml aprotinin. Blood was transferred to pre-cooled polystyrene tubes containing 1 mg/ml sodium EDTA. The tubes were centrifuged at 4°C for 20 min at 5000 rpm, and the plasma removed and stored at −70°C. In a preliminary study, blood was drawn from 17 hypertensive patients displaying elevated blood pressure at the time of examination and 13 normotensive control subjects.

2.5. *Plasma extraction*

Human plasma and rat plasma interfered nonspecifically with the RIA yielding falsely high levels of 'immunoreactivity'. Therefore, we developed an extraction method to exclude nonspecific interference and allow for the prepurification of ir-α-hANF. Amberlite XAD-2 adsorbent resin (particle size 0.3–1.0 mm; Serva, Heidelberg) was found to be most satisfactory, since it adsorbed more than 98% of synthetic α-hANF from plasma, independent of concentration in the range 6–200 fmol/ml. 9 g was packed into 10-ml plastic columns, 5 ml plasma was loaded onto the column, the columns rinsed with distilled water and ANF eluted with 9 ml of 55% acetonitrile in 0.1 M acetic acid. Eluates were lyophilized and either redissolved in buffer for RIA or subjected to HPGPC and subsequent HPLC analysis.

2.6. *HPGPC and reverse-phase HPLC*

HPGPC of plasma extracts was performed on 2 serially connected TSK-125 Bio Sil columns (7.5 × 600 mm) (Bio-Rad, Richmond, CA) eluted with 0.09% trifluoroacetic acid (TFA), containing 0.005 M Na_2SO_4 plus 0.002 M NaH_2PO_4 and 30% acetonitrile, as a solvent. The flow rate was 0.8 ml/min. Aliquots from column fractions were analyzed for ir-α-hANF as shown in fig.2. Thereafter, the immunoreactive peak co-eluting with synthetic standard was re-chromatographed on a Waters C18 μBondapack reverse-phase column (3.9 × 300 mm) and fractions assayed for α-hANF immunoreactivity (fig.3). The HPLC column was eluted with 0.1% TFA and a linear gradient of acetonitrile from 10 to 35%.

3. RESULTS

A typical standard curve for α-hANF and crossreactivities of the Toni II antiserum to α-rANF, AP I,II,III are shown in fig.1. The serum displays a high avidity for α-hANF. The detection limit of the RIA (90% displacement) was 7 fmol/tube. The antiserum also recognizes α-rANF and AP III with 50 and 70% lower avidity,

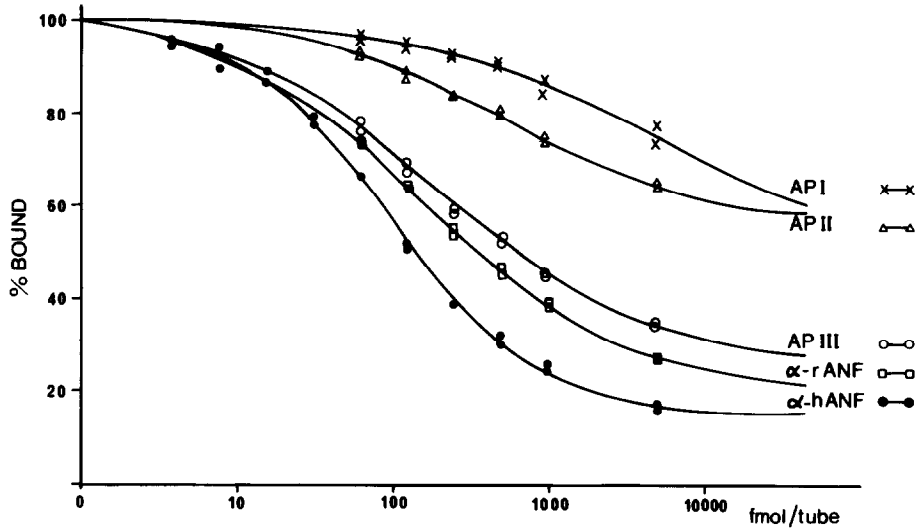

Fig.1. Typical standard curve of α-hANF and cross-reactivities of α-rANF and AP I, II, III in the RIA.

respectively. C-terminally abbreviated fragments, AP I and II, crossreact with the antiserum by 1 and 2%, respectively. Thus, for recognition by the Toni II antiserum an intact C-terminus is essential. Toni II showed negligible crossreactivity (<0.001%) with β-endorphin, dynorphin B, dynorphin A, dynorphin A_{1-8}, α-neo-endorphin, amidorphin, bovine-adrenal-medullary-22-peptide, *N*-acetyl-β-endorphin, Leu-enkephalin, ACTH, arg^8-vasopressin, oxytocin, angiotensin II, Met-Lys-bradykinin, and dopamine. To preclude false positive values due to tracer degradation in the RIA incubation, the complete plasma extract RIA mixture was subjected to reverse-phase HPLC on a C18 μBondapack column. All radioactivity eluted in the position of the non-incubated tracer. Since even a few microliters of un-extracted plasma in the RIA mixture caused gross, non-parallel inhibition of tracer binding, all plasma samples were extracted prior to RIA. As extraction by adsorption to XAD 2-adsorbent resins was performed with 5 ml plasma the detection limit was ~1.4 fmol/ml plasma. If necessary, the method allows for extraction of up to 15 ml plasma, yielding an effective increase in sensitivity such that 0.5 fmol/ml plasma can be detected. The efficiency of extraction (recovery) as determined by the addition of synthetic standards to 5-ml plasma aliquots was greater than 98% and independent of concentration in the range 6–200 fmol/ml. Levels of ir-α-hANF in normotensive subjects (n = 13) were found to be 8.9 ± 2.4 fmol/ml (mean ± SE). In contrast, considerably higher levels were seen in hypertensive patients (62.2 ± 19.7 fmol/ml, mean ± SE, n = 17) ($P \leq 0.02$, Student's two-tailed t-test).

4. DISCUSSION

This paper describes the development of an RIA for α-hANF which, in conjunction with a novel extraction and purification procedure employing adsorbent resins, allows for the detection of ir-α-hANF in human plasma in concentrations as low as 0.5 fmol/ml. This very high sensitivity appears to be particularly crucial in view of the very low levels, in the range 1–23 fmol/ml, of ir-α-hANF which were found in normotensive individuals. Miyata et al. [11] have, in fact, reported considerably higher levels in unextracted plasma. However, as for a great variety of peptide hormones [10,12,13] our studies suggest that these reflect non-parallel inhibition of tracer binding by plasma in the RIA for α-hANF, yielding misleadingly high values. Indeed, it appears that extraction is an essential step for the reliable determination of ir-α-hANF in human plasma. Analysis of plasma extracts on HPGPC (fig.2) and subsequent HPLC (fig.3) revealed that ir-α-hANF in human plasma is comprised, virtually exclusively,

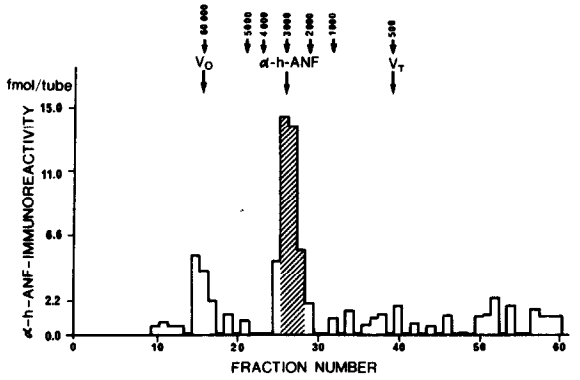

Fig.2. M_r pattern of ir-α-hANF in human plasma extracts on a TSK-125 Bio Sil HPGPC system. Fraction aliquots were assayed for α-hANF immunoreactivity. Columns were calibrated with V_o = bovine serum albumin, V_T = Leu-enkephalin and a series of opioid peptides and synthetic α-hANF.

of the authentic C-terminal 28-amino-acid residue portion of the precursor, human prepro-ANF [9]. Although the Toni II antiserum would appear capable of recognizing related ANF peptides with an intact C-terminus, no such α-hANF-related fragments were seen: however, the presence of small amounts of these below the limit of assay sensitivity cannot be discounted. Notwithstanding the occurrence of the N-terminally extended fragment containing α-hANF (γ-hANF, the 126-amino-acid residue peptide) in human hearts [14], we detected neither this nor other larger precursor forms in human plasma upon chromatography. This is in good agreement with a recent study in the rat in which it was indicated that the rat heart does contain high-M_r forms but secretes exclusively ANFs of 20–35 amino acid residues [15]. The development of such a highly sensitive assay allowing for the detection of authentic α-hANF in human plasma renders the evaluation of any possible change in α-hANF in pathological states feasible. In view of the ability of exogenously administered ANFs to lower the blood pressure [16], it is of interest that in a heterogenous group of hypertensive patients levels of ir-α-hANF in plasma were higher than in normotensive subjects. The mechanism underlying this increase is at present unclear: the attractive possibility that it reflects a (compensatory) accelerated release of ANF evidently requires further evaluation. Nevertheless, by use of this assay, it is clearly possible to evaluate the patterns of release of ANF and its molecular distribution in various forms of hypertension and cardiovascular disease. We are currently addressing these and other questions of clinical relevance.

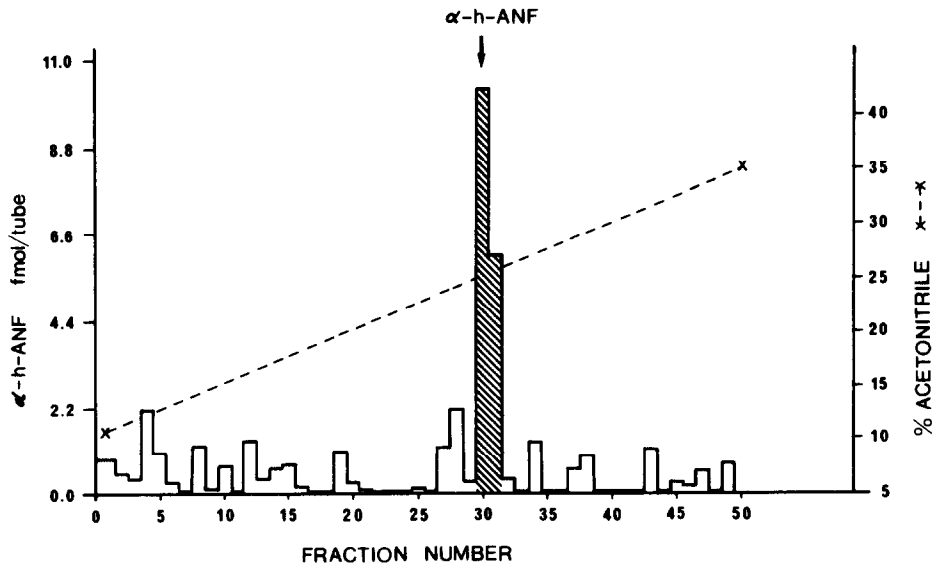

Fig.3. HPLC analysis of α-hANF from human plasma following HPGPC (see fig.2). The immunoreactive peak co-eluting with synthetic α-hANF on the HPGPC system was re-chromatographed on a Waters HPLC C18 reverse-phase column, and then re-assayed for α-hANF immunoreactivity.

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