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α-h-ANP is the only form of circulating ANP in humans

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α-human atrial natriuretic peptide (α-h-ANP) was purified to homogeneity from human plasma of healthy adults in a three-step procedure including immunoaffinity chromatography on immobilized monoclonal anti-α-h-ANP antibody (moab). A single peak of immunoreactivity was obtained after final reversed-phase HPLC and the amino acid sequence of the isolated material was identical to that of synthetic α-h-ANP. No further atrial peptides could be detected although the moab reacts with all biologically active peptides. It is therefore concluded that α-h-ANP is the only form of ANP circulating in human plasma.

Atrial natriuretic peptide; Immunoaffinity chromatography; Amino acid analysis; (Human plasma)

1. INTRODUCTION

Atrial natriuretic peptides (ANP) are a family of peptides possessing natriuretic, diuretic, and vasorelaxant properties [1]. Furthermore, inhibition of aldosterone synthesis [2] and renin release have been well documented [3]. The peptides or probably their precursors are stored in specific secretory granules in mammalian atrial cardiocytes [4]. ANP are secreted into the blood stream, the extension of the atria being the only known stimulus so far [5]. They have been isolated and sequenced from atria of several mammalian species (e.g. man, rat, bovine) [6]. The predominant low-M_r species purified from human atrial is α-human ANP (α-h-ANP). So far there exists only indirect evidence as to whether this is the only circulating form of ANP [8–11]. Here, we describe the purification of atrial peptides from human plasma by immunoaffinity chromatography and reversed-phase HPLC. The isolated material was homogeneous in nature and sufficient for amino acid sequence analysis.

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2. MATERIALS AND METHODS

Synthetic atrial peptides, rat ANP fragments (13–28) and (18–28), and human ANP fragment (7–28) were purchased from Novabiochem (Switzerland) or Peninsula (USA).

^{125}I-labelled rat ANP (1–28) was obtained from Amersham (England) or by iodination of rat ANP (1–28) using the chloramine T method [7]. Leu-enkephalin, [Ile^5]angiotensin II, [Arg^8]vasopressin, substance P, γ-MSH, and bovine serum albumin were from Sigma (FRG). Bradykinin, ACTH (porcine), aldosterone and insulin (rat) were from Serva (FRG).

2.1. *Generation of monoclonal antibodies*

α-h-ANP was coupled to keyhole limpet hemocyanin (KLH, Pacific Biomarine Supply, USA) using 0.25% glutaraldehyde. Female Balb/C mice (Moellegard, Denmark) were injected with 100 μg of antigen once in complete Freund's adjuvant intraperitoneally, followed by a second intraperitoneal injection 2 weeks later. 3 days before

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cell fusion the animals were finally injected intravenously with 100 μg antigen in phosphate-buffered saline. Spleen cells of immunized mice were fused with the myeloma cell line X 63-AG 8.653 according to standard techniques [15]. Clone cells were used to produce ascitic fluid in male Balb/C mice [14].

2.2. *Radioimmunoassay*

Determination of ANP was performed in RIA buffer (0.02 M Na_2HPO_4/NaH_2PO_4, 0.15 M NaCl, 0.01% thimerosal, 0.1% gelatin, 0.01% BSA, 0.1% Triton X-100; pH 7.4). 50 μl ^{125}I-atriopeptin III (approx. 6000 cpm) was added to 350-μl samples in RIA buffer followed by 100 μl anti-α-h-ANP ascites fluid (dilution 1:2 Mio.). Samples were incubated for 16 h at 4°C. Separation of antibody-bound and free peptide was performed by adding 800 μl of a charcoal suspension (0.02 M Na_2HPO_4/NaH_2PO_4, 2% Norit-A, 0.02% dextran 60, 0.1% BSA, 10 mM Na_2EDTA; pH 7.4). The sample was vigorously mixed, allowed to stand for 20 min and finally centrifuged (5 min, 3600 × g). Radioactivity of the supernatant was determined in an LKB 1271 RIA gamma-counter.

2.3. *Plasma extraction*

10 l of human plasma containing approx. 0.8 μg ANP immunoreactivity were chilled immediately after collection. To prevent proteolytic degradation plasma was supplemented with EDTA, phenylmethylsulfonyl fluoride (PMSF), aprotinin and bacitracin.

RP 18 adsorbent resin (Merck, FRG) was added and the suspension stirred gently for 30 min. The supernatant was discarded and the reversed-phase material was washed with 2% acetonitrile (CH_3CN) in 0.1% trifluoroacetic acid (TFA) and 10% CH_3CN in 0.1% TFA. The peptide fraction was eluted with 50% CH_3CN in 0.1% TFA. Fractions of the washing and elution steps were lyophilized and assayed for immunoreactivity.

2.4. *Immunoaffinity chromatography*

Anti-α-h-ANP moab was purified from mouse ascites fluid by affinity chromatography on immobilized α-h-ANP. 35 mg purified moab were coupled to 7 ml CNBr-activated Sepharose 4B (Pharmacia, Sweden) according to the manufacturer's procedure. The RP 18 eluate containing ANP immunoreactivity was dissolved in 10 ml of 0.2 M Na_2HPO_4/NaH_2PO_4 (pH 7.2) and applied to the immunoaffinity column (1 × 7 cm) at 8 ml/h, washed with 0.1 M CH_3COOH and eluted with 0.1 M CH_3COOH/0.5 M NaCl. 4-ml fractions were collected and assayed for ANP immunoreactivity. Immunoreactive material was desalted on RP 18 cartridge and lyophilized.

2.5. *Reversed-phase chromatography*

Final purification was achieved by HPLC on RP 18 Ultrasphere ODS (Beckman Instruments, USA) using a gradient from 2% CH_3CN to 60% in 0.1% TFA over 30 min. Fractions of 0.5 ml were collected, assayed for immunoreactivity, and lyophilized.

2.6. *Amino acid sequence determination*

The peptide was solubilized in 30 μl TFA. The sample was applied to a glass-fiber filter which was pretreated with 3 mg polybrene. Sequence analysis was performed using a gas-phase protein sequencer from Applied Biosystems (USA) [13].

3. RESULTS AND DISCUSSION

3.1. *Properties of the 11A-A11 anti-α-h-ANP moab*

The moab shows complete crossreactivity with rat ANP I, II, and III, rat ANP (1–28) and human ANP fragment (7–28) while there is no inhibition of ^{125}I-ANP binding by rat ANP fragments (13–28), (18–28) and (1–11) (fig.1). These findings suggest that the moab recognizes an epitope including the disulfide bridge and the surrounding amino acids Cys^7 and Asn^{24}. Due to these properties all biologically active ANP species are equally well recognized. There is no crossreactivity of the moab employed with a variety of other circulating peptides and renal active hormones ([Ile^5]angiotensin II, Leu-enkephalin, substance P, aldosterone, ACTH [porcine], bradykinin, γ-MSH, insulin [rat], [Arg^8]vasopressin, renin [porcine]) up to the micromolar range.

3.2. *Purification procedure*

In the first purification step (RP 18 batch) all immunoreactive material was eluted with 50% acetonitrile (recovery 72%). The 11A-A11 moab

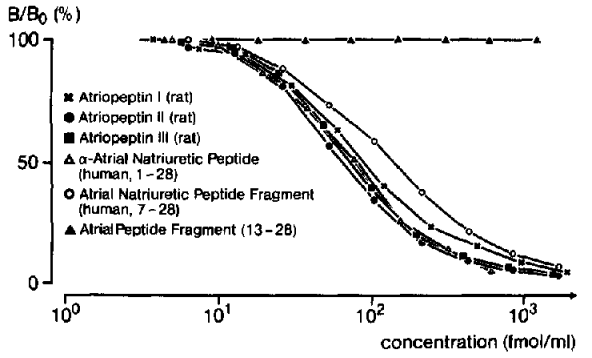

Fig.1. Crossreactivity of α-h-ANP (1–28) (△), r-ANP I (×), r-ANP II (●), r-ANP III (■), h-ANP fragment (7–28) (○), and h-ANP fragment (13–28) (▲) with ^{125}I-r-ANP III. Inhibition of binding was determined as described in section 2.

was used for affinity chromatography of the peptide extracts resulting in an 8000-fold purification in a single step (fig.2). Reversed-phase HPLC yielded final separation of ANP immunoreactivity from contaminating peptides (fig.3). The HPLC fractions (40 pmol) were then submitted to NH_2-terminal sequence analysis using a gas-phase sequencer. The sequence of α-h-ANP (1–28) was obtained.

Our findings are consistent with earlier reports suggesting that α-h-ANP is the circulating atrial peptide in humans [8–11]. This study, however, presents the first data on the amino acid sequence of material isolated from human plasma, whereas former conclusions concerning the nature of circulating ANP have only been drawn by comparison of retention times in reversed phase chromatography.

Besides α-h-ANP we did not find any other ANP species in blood of healthy adults. Several groups described the existence of circulating atrial peptides shorter than h-ANP [11,12]. In contrast to these results we could not identify any shorter forms of α-h-ANP although the crossreactivity of the anti-ANP moab would have allowed the detection of shorter forms of biologically active ANP [14].

Furthermore, no extended forms of α-h-ANP (pro-h-ANP) were detected although the moab also reacts with pro-h-ANP extracted from atrial

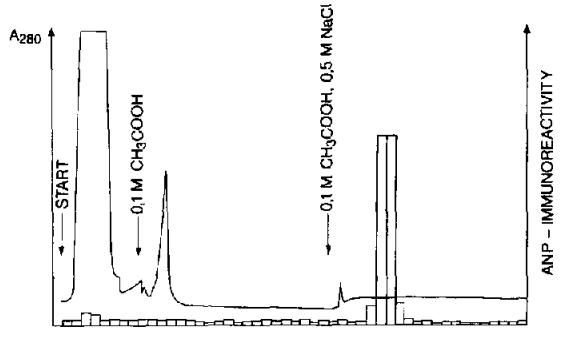

Fig.2. Affinity chromatography on anti-ANP-moab-Sepharose-4B. α-h-ANP-containing fractions of the RP 18 batch procedure were applied to the affinity column and eluted stepwise by 0.1 M CH_3COOH and 0.1 M CH_3COOH/0.5 M NaCl (flow rate 8 ml/h). Aliquots of each fraction were assayed for ANP immunoreactivity. Results are shown by bars. The continuous line indicates A_{280}.

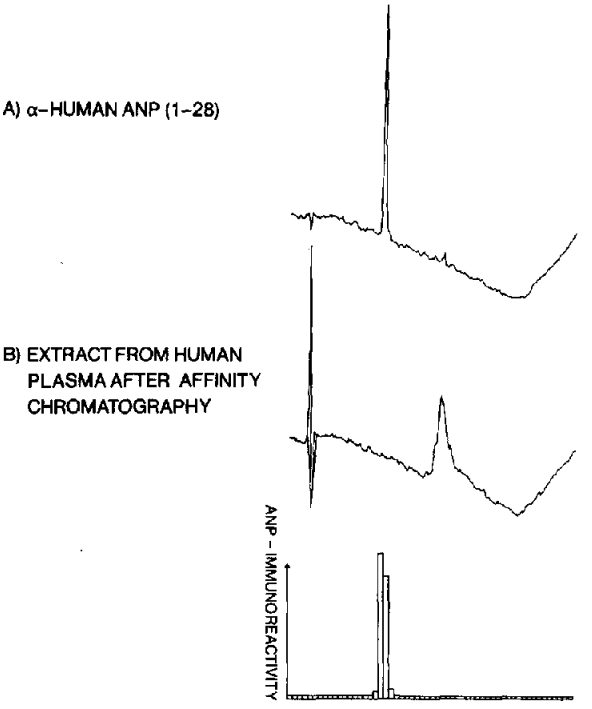

Fig.3. HPLC on RP 18. (A) A_{215} of 10 μg synthetic α-h-ANP (1–28). (B) The upper trace shows A_{215} of the affinity-purified α-h-ANP from human plasma. Aliquots of each 1 ml fraction were assayed for ANP immunoreactivity. Results are shown by bars. Flow rate: 1 ml/min. Solvent A: 0.1% TFA. Solvent B: acetonitrile gradient, 2–60% in 30 min.

tissue as demonstrated by Western blotting (not shown).

Assuming that ANP is stored in the atrial granula as a prohormone, the secretion mechanism as well as the proteolytic activating step await further investigation.

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