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FEBS Letters 341 (1994) 288–290

FEBS 13818

FEBS LETTERS

Immunoreactive adrenomedullin in human plasma

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Received 9 February 1994

Abstract

A specific and sensitive radioimmunoassay for adrenomedullin has been developed. Half-maximal inhibition of binding of radioiodinated adrenomedullin was observed at 4 fmol/tube. The radioimmunoassay recognized the entire adrenomedullin molecule and has little crossreactivity with adrenomedullin fragment peptides. Adrenomedullin-like immunoreactivity was found to circulate in human plasma at considerable concentration (3.3 ± 0.39 fmol/ml). The immunoreactivity of adrenomedullin was eluted at almost the same position as synthetic adrenomedullin on gel-filtration chromatography and reverse-phase high-performance liquid chromatography, suggesting that circulating adrenomedullin recognized by the present radioimmunoassay is identical or very similar to authentic adrenomedullin. Plasma immunoreactive adrenomedullin significantly increased in patients with hypertension, with a progressive rise proportionate to disease severity.

Key words: Adrenomedullin; Radioimmunoassay; High-performance liquid chromatography; Human plasma

1. Introduction

Adrenomedullin (AM) is a hypotensive peptide recently discovered by monitoring the elevating activity of platelet cAMP in human pheochromocytoma [1]. The peptide, consisting of 52 amino acids, has one intramolecular disulfide bond and shows slight homology with the calcitonin gene-related peptide. AM elicited a potent and long lasting hypotensive effect when injected intravenously in anesthetized rats [2]. Sequence analysis of cloned human AM cDNA showed that human AM precursor is 185 amino acids in length, including a putative signal peptide [3]. Studies of regional distribution in human tissue showed that immunoreactive (ir)-AM was abundant in the normal adrenal medulla as well as in pheochromocytoma tissue [1,4]. Ir-AM is present in circulating blood in considerable concentration [1]. These findings indicate that AM may be a new hormone participating in circulation control.

In order to investigate AM's physiological and clinical roles, it is essential to establish a radioimmunoassay (RIA) to measure its plasma concentration. In the present study, we established a highly sensitive and specific RIA for AM. With this RIA, we examined the concentration and molecular form of ir-AM in human plasma.

2. Materials and methods

2.1. Chemicals

Human AM, AM[13–52], AM[40–52], AM[1–13] and AM[13–31] were prepared by the solid phase method. Bovine serum albumin (BSA, Sigma) for RIA was pretreated with 5 mM *N*-ethylmaleimide for 24 h at room temperature and was purified by the ammonium sulfate precipitation method [5].

2.2. Preparation of antiserum for AM

Synthetic AM solution prepared 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 interscapulovertebral region.

2.3. Radioiodination

AM (10 μg) in 25 μl of 0.4 M sodium acetate buffer (pH 5.6) was introduced into a tube followed by the addition of $Na^{125}I$ (1.0 mCi/10 μl, Amersham). Lactoperoxidase (Calbiochem: 600 ng/10 μl of 0.1 M sodium acetate, pH 5.6) and H_2O_2 (70 ng/5 μl of water) were added to the reaction tube. After standing for 15 min at 30°C, H_2O_2 (100 ng/5 μl of water) was added to the tube, which was left for another 10 min at 30°C. Immediately after reaction, the mixture was submitted to reverse-phase high-performance liquid chromatography (HPLC), and monoiodinated AM was purified and used as a tracer.

2.4. Procedures for RIA

The incubation buffer for RIA was 0.05 M sodium phosphate buffer (pH 7.4), containing 0.5% BSA, 0.5% Triton X-100, 0.08 M NaCl, 0.025 M EDTA 2Na, 0.05% NaN_3, and Trasylol 500 KIU/ml. A disposable plastic tube (10 × 75 mm) was used for assay. All assay procedures were performed at 4°C. The standard AM or the unknown sample (100 μl) was incubated with anti-AM antiserum diluent (200 μl) for 12 h, then the tracer solution (18,000–20,000 cpm in 100 μl) was added. After incubation for 36 h, anti-rabbit IgG goat serum diluent (100 μl) was added. After standing for 24 h, the tubes were centrifuged at 2,000 × *g* for 30 min at 4°C and radioactivity of the precipitate was measured in an Aloka ARC-600 gamma counter.

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SSDI 0014-5793(94)00203-8

2.5. Preparation of plasma sample

Human plasma samples were taken with EDTA 2Na (1 mg/ml) and Trasylol (500 KIU/ml). Plasma was separated by centrifugation at 4°C and stored at −30°C until use. Five ml of plasma was acidified with 60 μl of 1 M HCl and diluted with 5 ml of saline and then loaded onto a Sep-Pak C18 cartridge (Waters) which was preequilibrated with saline. After washing with 5 ml of saline and 5 ml of 20% acetonitrile containing 0.1% trifluoroacetic acid (TFA), the adsorbed materials were eluted with 4 ml of 50% acetonitrile containing 0.1% TFA. The eluate was lyophilized, and the residual materials were dissolved in RIA buffer and the clear solution was submitted to RIA.

2.6. Statistics

Values are given as mean ± S.E.M. Statistical analysis was carried out using one-way analysis of variance with Bonferroni correction [6] for multiple group comparisons.

3. Results and discussion

3.1. RIA for AM

The antiserum to AM recognized the peptide with high affinity at a final dilution of 1:24,000. As shown in Fig. 1, half-maximum inhibition of radioiodinated ligand binding by AM was observed at 4 fmol/tube. From 0.5 to 32 fmol/tube of AM was measurable by this RIA system. The intra- and inter-assay coefficients of variance were 5% and 8%, respectively. This RIA had 100% crossreactivity with the Met sulfoxide form of AM, but had 2% and 0.5% crossreactivity with AM[13–52] and AM[40–52], respectively. It had less than 0.01% crossreactivity with AM[1–12] and AM[13–31]. These data indicate that this antibody recognizes the entire AM molecule.

The high specificity and sensitivity of the RIA for AM prepared above enabled us to determine the AM plasma concentration in healthy volunteers and patients with several disorders. However the plasma sample had to be treated with a Sep-Pak C-18 cartridge (Waters), since the plasma concentration of ir-AM in human plasma was found to be low.

Prior to measurement of ir-AM, the efficiency of extraction and reliability of RIA procedures were validated by the following experiments with normal human plasma:

(1) [^{125}I]AM or synthetic AM added to the plasma sample before extraction were reproducibly found to be more than 90% recovered when it was treated with a Sep-Pak C-18 cartridge.

(2) Dilution of RIA sample, prepared as described in section 2, yielded competition curves that were roughly parallel to the standard curves of AM as shown in Fig. 1.

(3) An appropriate amount of cold synthetic AM added to the RIA sample proved to be precisely determined by the present RIA.

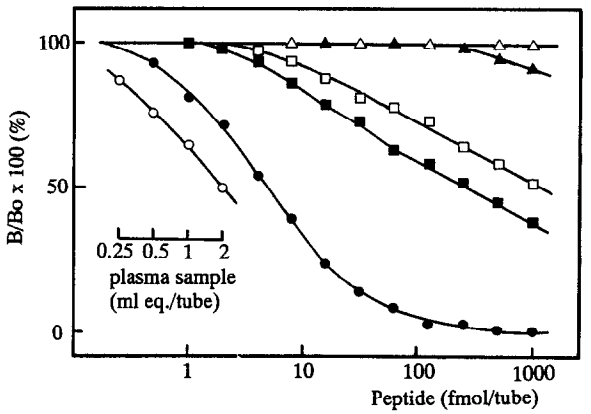

Fig. 1. Inhibition of [^{125}I]AM binding to antiserum against AM. (●), AM; (■), AM[13–52]; (□), AM[40–52]; (▲), AM[1–12]; (△), AM[13–31]. The dilution curves of plasma sample (○) are roughly parallel to those of standard AM.

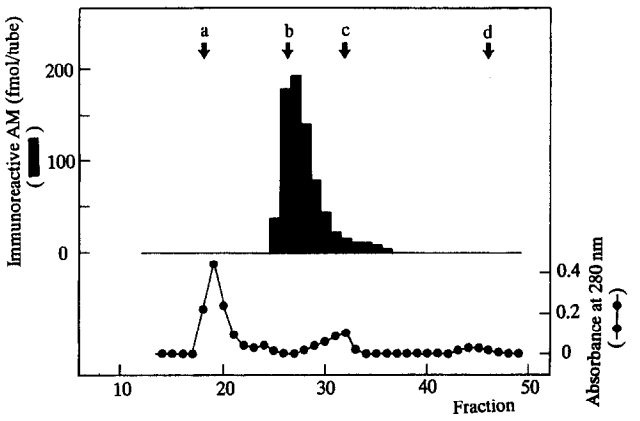

Fig. 2. Sephadex G-50 gel-filtration of human plasma sample monitored by RIA for AM. Sample: 100 ml of human plasma treated with Sep-Pak C18 cartridge as described in the text. Column size: 1.5 × 100 cm. Fraction size: 4.0 ml/tube. Elution buffer: 1 M CH_3COOH. Arrows indicate elution positions of: (a) void volume; (b) AM; (c) aprotinin; (d) NaCl.

3.2. Characterization of ir-AM in human plasma

Prior to assay of ir-AM in human plasma, the immunoreactive species present in human plasma were characterized by the following experiments. One hundred ml of healthy human plasma was treated with a Sep-Pak C18 cartridge as described in section 2. The sample thus obtained was submitted to gel filtration on a Sephadex G-50 column, and monitored by RIA for AM (Fig. 2). Only a single major peak of ir-AM was observed at a position almost identical with that of authentic human AM. The ir-AM fraction thus obtained was submitted to HPLC on a reverse phase column that was also monitored by RIA for AM. As seen in Fig. 3, one major peak of ir-AM, possessing more than 90% of the total immunoreactivity, emerged at a position almost identical with that of authentic human AM. These data indicate that ir-AM in human plasma, as monitored by the present RIA, is identical or very similar to authentic AM.

3.3. Ir-AM concentration in plasma of hypertensive patients

The high specificity and sensitivity of the RIA for AM enabled us to determine the plasma concentration of ir-AM in healthy volunteers and in patients with several disorders. In the present study, blood samples were drawn in the early morning from eight healthy volunteers. Furthermore, as a pathological condition in which plasma AM level might be increased, essential hypertension was focused, and blood samples were obtained from 19 patients with hypertension. The patients were grouped according to the World Health Organization (WHO) classification on the basis of their clinical characteristics. As shown in Fig. 4, in normal subjects the mean level of AM immunoreactivity in peripheral plasma was 3.3 ± 0.39 fmol per mililiter. The plasma ir-AM concentration in severe hypertension (WHO stage III) patients (8.39 ± 0.39) was significantly ($P < 0.05$) higher than that in normal subjects and mild hypertension subjects (4.66 ± 0.59). In addition, the mean plasma ir-AM concentration in mild and moderate hypertension (WHO stage I and II) showed a tendency to increase in comparison to that in normal subjects. Thus, the augmentation of plasma ir-AM in patients with hypertension seems to be progressive, proportionate to disease severity.

We have already shown [1,2] that AM elicited a potent and long lasting hypotensive activity which is comparable to that of CGRP, one of the strongest vasorelaxants known [7]. The potent vasodilator effect of AM has recently been shown to be a causative hypotensive mechanism in anesthetized rats [2,8]. Therefore, whatever the mechanism for the increase of AM in hypertension, hypotensive and vasodilator effects of AM should be expected to counteract with arteriolar vasoconstriction found in hypertension and may therefore be of benefit in vivo.

We have already shown that a high level of AM mRNA, found in lung, ventricle and kidney, as well as in adrenal medulla, may contribute to ir-AM in human plasma [3]. Although the souce of circulating AM remains obscure, the present data suggest that AM is a circulating hormone participating in circulation control. In addition, measurement of the plasma concentration of AM by the present RIA will help to clarify the pathophysiological role of AM in cardiovascular diseases.

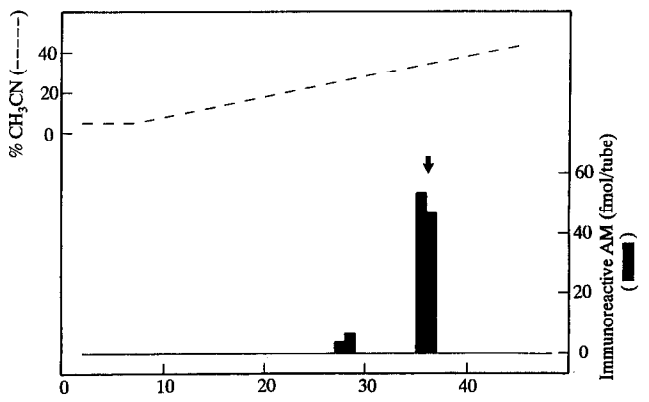

Fig. 3. Reverse-phase HPLC of plasma sample monitored by RIA for AM. Sample, half of fraction 35–38 in Fig. 2; Column, TSK ODS SIL 120 A (4.6 × 150 mm, Tosoh); solvent system, H_2O/CH_3CN/10%TFA = (I) 90:10:1, (II) 40:60:1 (v/v). Linear gradient from (I) to (II) for 60 min. Flow rate: 1 ml/min. The arrow indicates the elution position of AM.

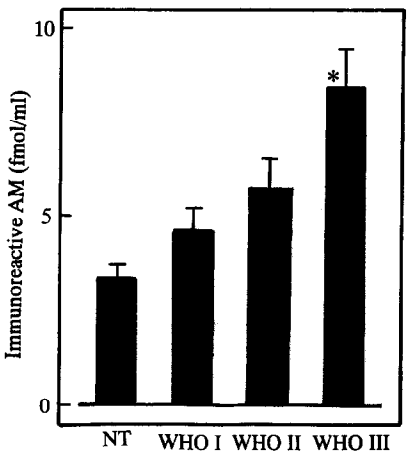

Fig. 4. Immunoreactive AM in plasma of normotensive (NT) volunteers and hypertensive patients. Patients were grouped according to WHO stages I–III on basis of clinical characteristics. Number of samples: NT, 8; WHO I, 6; WHO II, 8; WHO III, 5. *$P < 0.05$ vs. NT and WHO I.

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