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Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma

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Abstract

A specific and sensitive radioimmunoassay for human adrenomedullin has been developed and distribution and characterization of immunoreactive adrenomedullin in human tissue were investigated. The radioimmunoassay specifically recognizes its carboxyterminal region and half maximal inhibition of binding of radioiodinated adrenomedullin(40–52)NH_2 was observed at 11 fmol/tube. Immunoreactive adrenomedullin was abundant in adrenal medulla (47.7 ± 26.1 fmol/mg, mean ± S.D.) and was ubiquitously found in all tissue examined. The mean plasma concentration of adrenomedullin in three normal individuals was 17.2 ± 6.4 pg/ml (mean ± S.D.). By analysis with reverse-phase high-performance liquid chromatography coupled with the radioimmunoassay, most immunoreactive adrenomedullin in the adrenal medulla, atrium and lung was found to be adrenomedullin(1–52)NH_2

Key words: Adrenomedullin; Specific radioimmunoassay; Distribution; Characterization

1. Introduction

Adrenomedullin (AM) is a potent hypotensive peptide which has been recently identified in extracts of human pheochromocytoma (PC) tissue by monitoring its elevating activity on rat platelet cAMP. AM, consisting of 52 amino acid residues, has a C-terminal tyrosine amide structure and one intramolecular disulfide bond, and shares slight homology with calcitonin gene-related peptide (CGRP) and amylin [1]. The hypotensive activity of AM is comparable to that of CGRP which has been established as one of the strongest vasorelaxants [2].

In our former study, we developed a radioimmunoassay (RIA) for AM(3–12), which is generated from AM and its precursor by trypsin digestion. However, this RIA could not recognize AM(1–52)NH_2 without trypsin digestion. AM was found to be abundant in human adrenal medulla as well as in PC [1], while high levels of AM mRNA were found in lung, ventricle and kidney as well as in adrenal medulla and PC [3]. To elucidate the biosynthesis of AM in several tissues and to investigate the possible biological role of AM, it is essential to develop an assay system to detect AM. In the present study, we have established a specific and sensitive RIA for human AM and have determined the distribution and molecular form of AM in human tissue and plasma.

2. Materials and methods

2.1. Peptides

Adrenomedullin(40–52), (45–52), (47–52)NH_2 and (45–52)COOH were synthesized by the solid phase method with a peptide synthesizer (430A, Applied Biosystems), and purified by reverse-phase high-performance liquid chromatography (HPLC). Human synthetic adrenomedullin prepared by solid phase methods in the Peptide Institute, Inc. (Osaka, Japan), will be described elsewhere.

2.2. Preparation of antiserum

Human AM(40–52)NH_2 (10 mg) was conjugated with bovine thyroglobulin (20 mg) by the carbodiimide method [4]. The reaction mixture was dialyzed four times against one liter of 0.9% NaCl and two times against one liter of 0.05 M sodium phosphate buffer (pH 7.4), containing 0.08 M NaCl. The antigenic conjugate solution (1.5–3 ml) was emulsified with an equal volume of Freund's complete adjuvant, and was used for immunizing New Zealand white rabbits by subcutaneous injection. Animals received booster injections every two weeks and were bled 7 days after each injection.

2.3. Radioiodination of ligand

N-succinimidyl 3-(4-hydroxy,5-(^{125}I)iodophenyl)propionate was purchased from Amersham, Inc. (Tokyo, Japan). Human AM(40–52)NH_2 was radioiodinated by the Bolton and Hunter method [5]. The ^{125}I-labeled peptide was purified by reverse-phase HPLC on a TSK ODS 120A column.

2.4. Procedures for RIA

RIA for adrenomedullin was performed by a method similar to that

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Abbreviations: AM, adrenomedullin; RIA, radioimmunoassay; HPLC, high-performance liquid chromatography; ir-, immunoreactive; PC, pheochromocytoma; TFA, trifluoroacetic acid; BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; NPY, neuropeptide Y.

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reported for C-type natriuretic peptide [6]. Incubation buffer for RIA for adrenomedullin was 0.05 M sodium phosphate buffer (pH 7.4), containing 1% bovine serum albumin (BSA), 0.5% Triton X-100, 0.08 M NaCl, 0.025 M EDTA-2Na and 0.05% NaN_3. The RIA incubation mixture consisted of 100 μl of either the standard or the sample solution, 50 μl of antiserum at a dilution of 1:182,250 and 50 μl of ^{125}I-labeled ligand (18,000 cpm) in the standard buffer. The above mixture was placed in a plastic tube (7.5 × 78 mm), mixed well, and then equilibrated at 4°C for 24 h. The incubation was stopped by adding 50 μl of 1% bovine g-globulin and 500 μl of 24% polyethyleneglycol (#6,000) in the standard buffer. After vigorous shaking, the mixture was incubated at 4°C for 20 min and centrifuged at 2,000 × *g* at 4°C for 30 min. The supernatant was aspirated, and radioactivity in the pellets was counted in a gamma counter. Assays were routinely performed in duplicate.

2.5. Preparation of peptide extract

Peptide extract was prepared by a method similar to that previously described [7,8]. Human tissues were obtained from cadavers and human pheochromocytoma tissues were resected at surgery from three norepinephrine dominant pheochromocytoma patients. Diced pieces of adrenal medulla and pheochromocytoma tissues were boiled for 10 min in 3 vol. of 1 M acetic acid. Those of the other tissues were boiled for 10 min in 3 vol. of H_2O to inactivate intrinsic proteases. After cooling, glacial acetic acid was added to a final concentration of 1 M. The mixture was homogenized with a polytron mixer at 4°C. The extract supernatant was obtained after 30 min centrifugation at 24,000 × *g*. The supernatant from each tissue was loaded to a Sep-Pak C-18 cartridge (Waters, Inc., Massachusetts, USA) pre-equilibrated with 0.5 M acetic acid. Adsorbed materials were eluted with 3 ml of 60% acetonitrile in 0.1% trifluoroacetic acid (TFA). The eluates were evaporated in vacuum to dryness. Residual materials were dissolved in RIA buffer and the clear solution submitted to RIA.

3. Results and discussion

3.1. RIA for AM

The antiserum against AM was raised by injection of synthetic human AM(40–52)NH_2 conjugated with thyroglobulin. Antiserum designated #172CI-9 recognized human AM(40–52)NH_2 with high affinity at a final dilution of 729,000. As seen in Fig. 1, half-maximal inhibition by AM(40–52)NH_2 and human AM was observed at 11 fmol/tube, and the peptide was detectable as low as 1 fmol/tube. The intra- and inter-assay coefficients of variance were less than 6% and 9%, respectively. The dilution curve of extracts of human adrenal medulla was parallel to the standard curve. Human AM, AM(45–52)NH_2 and AM(47–52)NH_2 showed 100% cross-reactivity with the antiserum, but human AM(45–52)COOH showed only 2.5% cross-reactivity. In addition, ^{125}I-Tyr^{52}AM(40–52)NH_2 radioiodinated by the lactoperoxidase method [9] was poorly recognized by antiserum #172CI-9. Therefore, antiserum #172CI-9 specifically recognized mainly the C-terminal tyrosine amide structure of AM. These results indicate that this RIA may be useful for detection of biological active AM, because of the importance of the C-terminal amide structure in many biologically active peptides [10]. Although AM shares slight homology with CGRP or amylin, the antiserum did not exhibit cross-reactivity with human CGRP-I, II and amylin. Neuropeptide Y (NPY) also has a C-terminal tyrosine amide structure, but the present RIA showed no cross-reactivity with NPY.

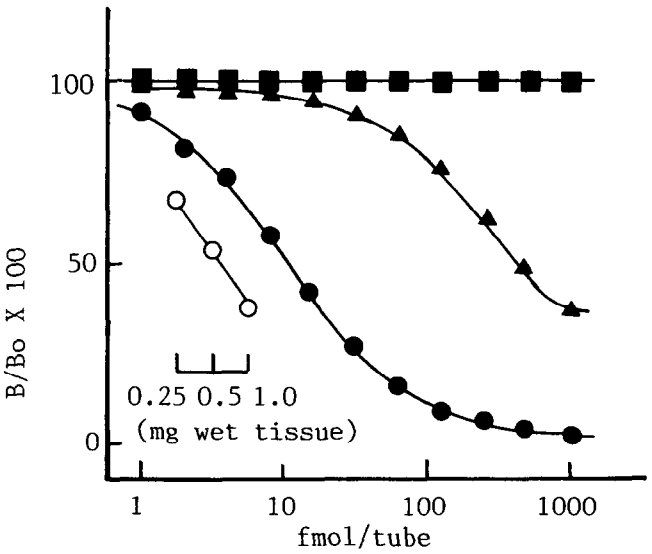

Fig. 1. Standard curve of radioimmunoassay for adrenomedullin and cross-reactivity of antiserum #171CI-9. Inhibition of ^{125}I-labeled AM(40–52)NH_2 binding to the antiserum by serial dilutions of AM(1–52)NH_2, (45–52)NH_2, (47–52)NH_2 (●), (45–52)COOH (▲), CGRPs (■), amylin (■) and NPY (■). AM(45–52)COOH showed 2.5% cross-reactivity with the antiserum. The latter three were derived from human and none exhibited any cross-reactivity with the antiserum. Inhibition of ^{125}I-labeled AM(40–52)NH_2 binding to the antiserum by serial dilutions of human adrenal medulla sample (○), is roughly parallel to that of standard AM(1–52)NH_2.

Table 1
Regional distribution and plasma concentration of immunoreactive adrenomedullin in human

Region	Immunoreactive adrenomedullin
Adrenal medulla	47.7 ± 26.1
Pheochromocytoma	92.1 ± 101.6
Heart Atrium	1.68 ± 1.58
Heart Ventricle	0.15 ± 0.02
Aorta	0.42 ± 0.09
Lung	0.80 ± 0.37
Kidney	0.35 ± 0.12
Pancreas	1.04 ± 0.35
Small intestine	0.97 ± 0.45
Liver	0.20 ± 0.06
Spleen	0.53 ± 0.10
Brain cortex	0.31 ± 0.15
Thyroid gland	0.28 ± 0.12
Plasma*	17.2 ± 6.4

Results are expressed as fmol/mg wet tissue.
*Plasma concentration of AM is expressed as pg/ml. All values are mean ± S.D. for three to four samples.

3.2. Distribution of ir-adrenomedullin in human tissue

Tissue AM contents are summarized in Table 1. High concentrations of immunoreactive (ir-) AM were present in adrenal medulla (47.7 ± 26.1 fmol/mg wet tissue, mean ± S.D.), confirming our previous results [1]. Fur-

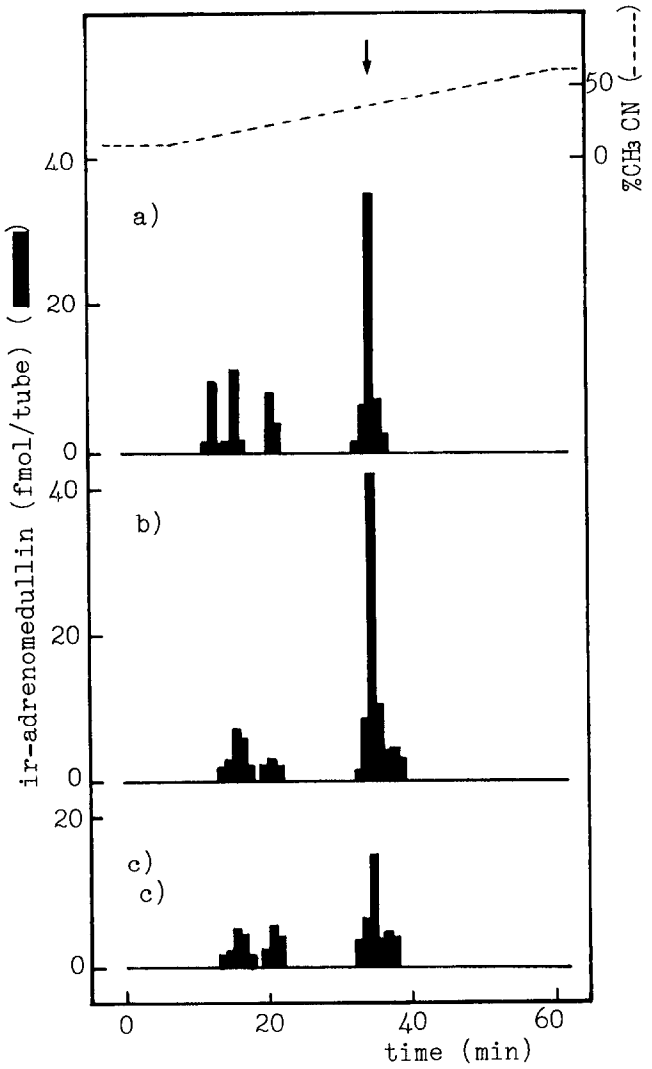

Fig. 2. Reverse-phase HPLC of human tissue extract monitored by RIA for AM. Sample: (a) adrenal medulla (2 mg eq.), (b) atrium (80 mg eq.), (c) lung (150 mg eq.). Column: TSK ODS 120A (4.6 × 150 mm, Tosoh). Flow rate: 1 ml/min. Solvent system: (A) H_2O/CH_3CN/10% TFA = 90:10:1 (by volume), (B) H_2O/CH_3CN/10% TFA = 40:60:1 (by volume). Linear gradient from (A) to (B) for 60 min. The arrow indicates elution position of AM.

thermore, ir-AM was also detected in atrium, lung, pancreas and small intestine in relatively high concentrations and was found in smaller amounts in brain and ventricle. The concentration of ir-AM in ventricle, kidney and lung was less than 3% of that in adrenal medulla, while high levels of AM mRNA were found in ventricle, kidney and lung as well as in adrenal medulla [3]. This discrepancy may be explained by the possibility that AM which is biosynthesized in these tissues may be rapidly released into the blood or metabolized in each tissue. Although ir-AM was detected in pancreas and small intestine, very slight AM mRNA expression was detected in pancreas and intestine [3], suggesting that biosynthetic systems and excretion may be different in each tissue. In addition, it is interesting that ir-AM concentration in atrium was more than 10-fold that in ventricle. Even though the physiological implication of AM in atrium is obscure, it may be involved in circulatory control in a similar manner to that of CGRP and ANP which were found to be abundant in atrium [11,12].

PC ir-AM concentration was 92.1 ± 101.6 fmol/mg wet weight (mean ± S.D.), which is the highest concentration among all tissues examined. Although ir-AM concentration of PC varied in each case, PC may be a tumor that produces AM as well as catecholamine.

The mean plasma concentration of AM in three normal individuals ranged from 12.2 to 24.4 pg/ml with a mean of 17.3 ± 6.4 (mean ± S.D.) pg/ml.

3.3. *Characterization of ir-AM in adrenal gland, atrium, lung and plasma*

The ir-AM in adrenal gland, atrium and lung was further characterized by reverse-phase HPLC using a TSK ODS 120A column coupled with RIA for AM. As shown in Fig. 2, one major peak and several minor peaks of ir-AM were observed in adrenal gland, atrium and lung. The major peak emerged at an identical elution time with authentic AM(1–52)NH_2. Furthermore, by gel filtration HPLC on a TSK-Gel 2000SW column (Tosoh, Inc. Japan) coupled with RIA for AM, in adrenal medulla the major ir-AM peak emerged at an identical elution time with authentic AM as shown in Fig. 3. The minor ir-AM peak which emerged in the lower molecular weight range is considered to be C-terminal fragments of

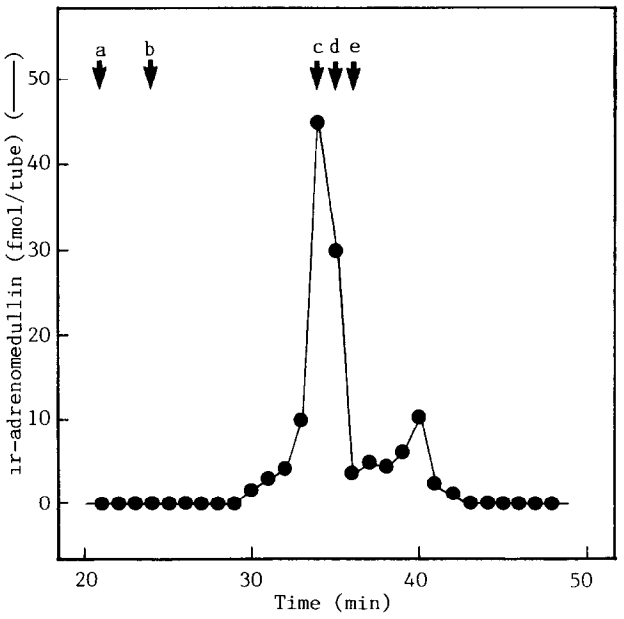

Fig. 3. Gel filtration HPLC of human adrenal medulla extract monitored by RIA for AM. Sample: adrenal medulla (10 mg eq.) Column: TSK-Gel 2000SW (8.0 × 500 mm, Tosoh). Eluent: 30% CH_3CN solution containing 0.1% TFA and 0.2 M NaCl. Flow rate: 0.5 ml/min. Arrows indicate elution positions of (a) Vo, (b) cytochrome *c*, (c) AM, (d) CGRP-I, (e) insulin chain B, respectively.

AM, as antiserum #172CI-9 recognized mainly the C-terminal tyrosine amide structure of AM. Although the major ir-AM peak was found to be AM itself, several minor peaks were observed in each of the three different tissues examined, the identification of which is now under way.

By gel filtration of human plasma coupled with RIA for AM, two major ir-AM peaks were observed as shown in Fig. 4. The first ir-AM peak, which constituted about 50% of the total ir-AM, emerged at an identical elution time with authentic AM. The second peak emerged in the lower molecular weight range. Two peaks were further characterized by reverse-phase HPLC coupled with RIA for AM. The first ir-AM peak in the gel filtration of plasma was eluted at an identical position with authentic AM and the second ir-AM peak was eluted at an earlier position (Fig. 5). These results indicate that ir-AM in human plasma consisted of AM itself and its C-terminal fragment whose biological activity remains obscure.

In conclusion, we have established a new specific and sensitive RIA for AM which recognizes the C-terminal tyrosine amide structure of AM. High concentrations of ir-AM were found in adrenal medulla and PC, where AM was originally discovered. In addition, ir-AM was found in all human tissues examined, indicating that AM

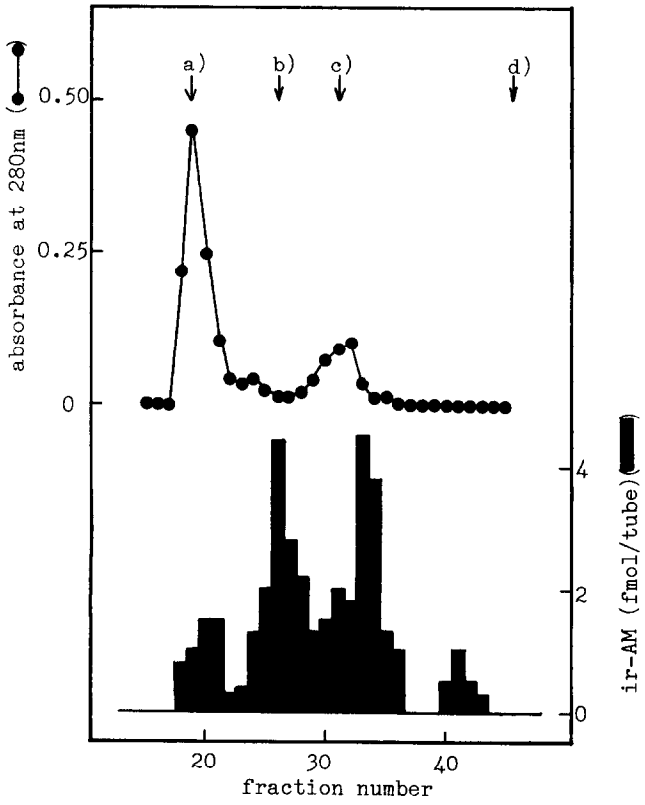

Fig. 4. Gel filtration of human plasma monitored by RIA for AM. Sample: human plasma (100 ml). Column: Sephadex G-50 (1.5 × 100 cm). Eluent: 1 N acetate. Flow rate: 12 ml/h. Arrows indicate elution positions of (a) Vo, (b) AM, (c) aprotinin, (d) Vt, respectively.

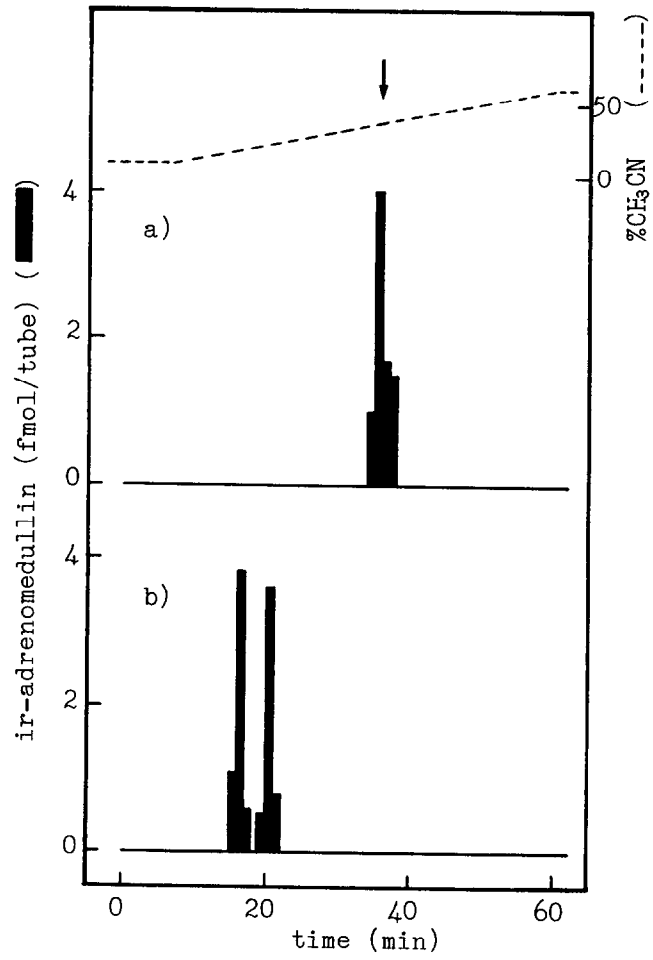

Fig. 5. Reverse-phase HPLC of two major peaks in Fig. 4 monitored by RIA for AM. Sample: (a) first major peak, (b) second major peak in Fig. 4. Column: TSK ODS 120A (4.6 × 150 mm, Tosoh). Flow rate: 1 ml/min. Solvent system: (A) H_2O/CH_3CN/10% TFA = 90:10:1 (by volume), (B) H_2O/CH_3CN/10% TFA = 40:60:1 (by volume). Linear gradient from (A) to (B) for 60 min. The arrow indicates elution position of AM.

is ubiquitously distributed in human tissue and may be involved in circulatory control in these organs. Measurement of the plasma concentration of AM could help to clarify the pathophysiological significance of AM in a variety of diseases.

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