Autoradiographic Studies on the Binding Sites of ^{125}I -Adrenomedullin in Rat Tissues

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Adrenomedullin (AM) is a novel hypotensive peptide isolated from the human pheochromocytoma. In the present study, ^{125}I -AM was intravenously administered to rats and its distribution was examined by autoradiographic (ARG) techniques using whole-body, light microscopic (LM) and electron microscopic (EM) methods. Whole-body-ARG after administration of ^{125}I -AM showed the highest radioactivity in the lung (parenchyma), followed by the renal cortex and the liver. When localization of ^{125}I -AM in the lung was examined by LM-ARG, silver grains were localized over the arteries, capillaries and venules. In the arteries, the endothelial cells of the small arteries (lumen diameter

20–50 μm) were preferentially labelled and there was little labelling on the large arteries. There were few silver grains on the smooth muscle cells along the entire pulmonary vasculature system. EM-ARG showed many silver grains on the endothelial cells of capillaries and venules. Very few silver grains were observed on the lung when an excess amount of unlabelled AM was administered with ^{125}I -AM.

These results indicated that ^{125}I -AM specific binding sites are most abundant in the lung, and the major binding sites are on the endothelial cells of small arteries, alveolar capillaries and venules.

Key words: Adrenomedullin, Binding sites, Autoradiography, Lung, Endothelial cells

I. Introduction

A novel hypotensive peptide, named adrenomedullin (AM), was recently identified in the extract from human pheochromocytoma tissue by monitoring the elevated activity of platelet cAMP [5]. This peptide, consisting of 52 amino acid residues, has a structural homology with calcitonin gene-related peptide and amylin, and elicits a strong and long-lasting hypotensive effect when intravenously administered to experimental animals [3, 5]. A specific radioimmunoassay (RIA) showed AM to be present in the circulation in both rats and humans [2, 15]. AM mRNA is highly expressed in the adrenal medulla, lung, heart, and kidney in both rats and humans [4, 14].

AM has been shown to be synthesized and secreted by endothelial cells [18]. However, the target cells of AM remained to be completely identified.

In this study, we investigated the tissue binding sites of ^{125}I -AM in rats at the macro-, micro- and ultrastructural levels after administration of ^{125}I -AM by using whole-body-, LM- and EM-ARG.

II. Materials and Methods

Radioiodination of AM

Human-AM was purchased from the Peptide Institute Inc. (Osaka, Japan). AM was radioiodinated by the lactoperoxidase method [6]. AM (10 $\mu\text{g}/25\ \mu\text{l}$ of 0.4 M sodium acetate buffer, pH 5.6) was introduced into a tube followed by addition of Na^{125}I (37 MBq/10 μl , Amersham plc, UK). Lactoperoxidase (600 ng/10 μl of 0.1 M sodium acetate buffer, pH 5.6) and H_2O_2 (70 ng/5 μl of water)

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were added to the reaction tube, then left standing for 15 min at 30°C. Subsequently, H₂O₂ (100 ng/5 µl of water) was added to the tube, which was left standing for another 10 min at 30°C. Immediately after reaction, the mixture was subjected to reverse-phase HPLC, and monoiodinated AM was purified and used as a tracer. The specific radioactivity obtained was about 74000 GBq (2000 Ci)/mmol.

Animals

Male Sprague Dawley rats (Clea Japan Inc, Tokyo, Japan), weighing about 250–300 g, were used. The rats were kept on a complete pellet diet with free access to water.

Whole-body autoradiography

For whole-body-ARG, three rats received intravenous (i.v.) administration into the tail vein of 3.7 MBq (0.05 nmol)/kg of ¹²⁵I-labelled human-AM dissolved in phosphate-buffered saline (pH 7.2). They were sacrificed under ethyl ether anesthesia after 2 min. The animals were rapidly frozen by immersion into liquid nitrogen at –180°C and embedded in 3% carboxymethyl cellulose. Sagittal sections (40-µm thick) were cut using a cryomicrotome (PMV-450MP, Stockholm, Sweden) at –20°C. The sections were freeze-dried at –20°C for 2 days. The surface of the sections was covered with a protective membrane and placed in contact with X-ray film (Medical X-ray Film; Fuji Photo Film, Co., Ltd., Tokyo, Japan) from 2 to 5 weeks at room temperature. In a separate experiment, rats were administered ¹²⁵I-AM and an excess of unlabelled human-AM (5 nmol/kg), then sacrificed after 2 min for whole-body-ARG as described above.

Tissue distribution of ¹²⁵I-AM

Rats were i.v. administered ¹²⁵I-AM alone (0.37 MBq/0.005 nmol/kg; n=3) or ¹²⁵I-AM in the presence of an excess of unlabelled AM (5 nmol/kg; n=3). The animals were sacrificed after 2 min. After the blood was withdrawn, the lung, kidney, liver and thyroid were excised and weighed. The radioactivity of each tissue was measured with a gamma counter (ARC-600; Aloka, Tokyo, Japan).

Tissue preparation for light and electron microscopic autoradiography

For the light microscopic autoradiography (LM-ARG), three rats were killed under ether anesthesia at 2 min after i.v. administration of ¹²⁵I-AM alone (3.7 MBq/0.05 nmol/kg). In a separate experiment, a mixture of ¹²⁵I-AM and an excess of unlabelled human-AM (5 nmol/kg) was administered to one rat. The rat was sacrificed 2 min later. Blood was withdrawn and the whole lung was removed after infusing a fixative solution (2% formaldehyde, 0.5% glutaraldehyde, 0.1% tannic acid in 0.1 M cacodylate buffer, pH 7.2) through the

trachea. The whole lung was then immersed in fixative in a small beaker (100 ml). To avoid high temperature during the microwave irradiation (MWI) procedure which lasted for 30–35 sec, the beaker was put in a large petri-dish containing 200 ml water [9, 10]. After MWI, the tissue was cut into small pieces (0.5–1 cm³ blocks), put into the fixative in a small glass vial in the petri-dish, and again subjected to MWI for 30–35 sec. The kidney and liver were removed from the body as soon as possible, and cut into small pieces in a drop of fixative. These tissue blocks were put into the fixative in small glass vials in the petri-dish, then subjected to MWI for 30–35 sec. The tissue blocks were left in the glass vials with the fixative for 60 min at room temperature. The tissue blocks were dehydrated in serial ascending ethanol solutions and embedded in Technovit 7100 resin (Kulzer & Co., GmbH, Germany). Radioactivity was measured before fixation and after dehydration by a gamma counter, thus confirming the proper fixation of ¹²⁵I-AM in the tissue blocks.

For electron microscopic autoradiography (EM-ARG), two rats were sacrificed at 2 min after i.v. administration of ¹²⁵I-AM (11.1 MBq/0.15 nmol/kg). The lung, kidney and liver were fixed by MWI method as described above. After MWI, the tissue was cut into small pieces (1–3 mm³ blocks). The tissue blocks were post-fixed in 0.1 M cacodylate-buffered 1% osmium tetroxide, pH 7.2, at room temperature for 60 min, dehydrated in serial ascending ethanol solutions and embedded in Luvac 812 (Nacalai Tesque, Inc., Kyoto, Japan) in the usual manner.

Light microscopic autoradiography (LM-ARG)

The embedded tissue blocks (lung, kidney and liver) were cut into 1-µm-thick sections with a microtome (AS 500, Anglia Scientific, U.K.) using glass knives, mounted on glass slides and coated with photographic emulsion (NTB-3, Kodak Co., Ltd., Tokyo, Japan) by dipping method. They were exposed in dark-boxes for 6 weeks at 4°C and developed using Dektol (Kodak Co., Ltd., Tokyo, Japan) at 16°C for 4 min. The sections were stained with a mixture of methylene blue-basic fuchsin and observed with a light microscope (Optiphot, Nikon, Co., Ltd., Tokyo, Japan).

Electron microscopic autoradiography (EM-ARG)

Ultrathin sections were made from tissue blocks of the lung, kidney and liver. The sections were picked up on grids, stained with uranyl acetate and coated with carbon. Using the loop technique, L-4 emulsion (Ilford, U.K.) was applied to the grids. The samples were exposed in dark-boxes for 2 months at 4°C and developed by a modified phenidon method at 12°C for 1 min [12]. These grids were lead-stained and degelatinized at the same time, then observed with a transmission electron microscope (JEM 1200EX, JEOL, Tokyo, Japan).

Statistical analysis

The results are expressed as the mean ± SE. An un-

Fig. 1. Whole-body autoradiograms of rats administered ^{125}I -AM (**A**) alone and with an excess of unlabelled AM (**B**). The highest radioactivity was in the lung followed by the liver, renal cortex. After administration of an excess amount of unlabelled AM, the radioactive binding in the lung was substantially lower and the radioactivity in the liver was higher. The exposure lasted for 5 weeks. **A, B:** $\times 0.7$, Bar = 2 cm.

Fig. 2. Autoradiograms of the lung after administration of ^{125}I -AM (**A**) and ^{125}I -AM with an excess of unlabelled AM (**B**). The radioactivity of ^{125}I -AM was considerably reduced by simultaneous administration of an excess amount of unlabelled AM. The exposure lasted for 2 weeks. **A, B:** $\times 3.5$, Bar = 2 mm.

paired *t*-test was used to analyze the statistical differences. The means of two groups were considered significantly different at a value of $p < 0.05$.

III. Results

Whole-body autoradiography

Fig. 1A shows a whole-body autoradiogram for the rat sacrificed 2 min after administration of ^{125}I -AM alone. The highest radioactivity (density in X-ray films) was found in the lung (Fig. 1A; Lung, Fig. 2A), followed by the renal cortex and liver. Moderate levels of radioac-

tivity, which were comparable to the radioactivity of the blood in the left ventricle of the heart, were observed in the spleen, bone marrow and adrenal gland. The radioactivity levels in the thyroid, stomach, intestine and salivary gland were similar to those in the blood. In other organs, the radioactivity was less than that of the blood. The lowest levels of radioactivity were found in the central nervous system and eyeballs.

The whole-body autoradiogram of the rat, which was administered an excess of unlabelled AM (5 nmol/kg) together with the ^{125}I -AM, showed a substantial decrease in the amount of radioactivity in the lung (Fig. 1B; Lung,

Table 1. Tissue distribution of radioactivity at 2 min after administration of ^{125}I -AM or ^{125}I -AM with an excess of unlabelled AM.

Tissue	Radioactivity (dpm/mg wet tissue)	
	^{125}I -AM	^{125}I -AM + unlabeled-AM
Blood	17.4 $\pm$ 0.5	13.4 $\pm$ 2.0
Plasma	24.1 $\pm$ 1.2	19.8 $\pm$ 4.2
Lung	393.5 $\pm$ 7.4	295.8 $\pm$ 11.5**
Renal cortex	38.6 $\pm$ 4.2	28.6 $\pm$ 2.9
Liver	15.2 $\pm$ 1.0	28.3 $\pm$ 3.2*
Thyroid	24.1 $\pm$ 4.2	34.0 $\pm$ 5.3

Data are expressed as the mean $\pm$ s.e. of three animals.

* $p < 0.05$, ** $p < 0.01$ vs. group administered ^{125}I -AM alone (Student's *t*-test).

Fig. 2B) and a small increase in the liver. Radioactivity levels in all other organs and also in the blood were not affected.

Tissue distribution of ^{125}I -AM

Table 1 shows the radioactivity of the tissue (dpm/mg wet tissue), for the rat sacrificed 2 min after the administration of ^{125}I -AM with and without an excess of unlabelled AM (5 nmol/kg).

The accumulation of ^{125}I -AM in the lung was significantly ($p < 0.01$) attenuated by the simultaneous administration of an excess amount of unlabelled AM, but the radioactivity in the liver was significantly ($p < 0.05$) increased. In other tissues tested in this study, i.e. the blood, kidney and thyroid, the administration of unlabelled AM did not affect the amount of radioactivity.

Light microscopic autoradiography (LM-ARG)

The diffusion of the radioactivity during the preparation of tissue blocks was insignificant. The residual ratio of radioactivity after the preparation of the lung was $98.2 \pm 0.5\%$ ($n=3$), which showed that ^{125}I -AM was adequately fixed in the tissue.

The distribution of radioactivity was indicated by the presence of silver grains. In the lung, silver grains were observed over the vascular tree in the rat 2 min after administration of ^{125}I -AM alone. The silver grains were preferably localized on the endothelial cells of the small arteries (20–50 μm in diameter, Fig. 3A). However, there were very few grains on the large arteries (above 50 μm in diameter, Fig. 3B). Silver grains were also observed along the alveolar capillaries (Fig. 3C; arrow heads) and venules

(Fig. 3C; arrow, Fig. 3D). There were no silver grains on the smooth muscle cells along the entire pulmonary vascular tree (Fig. 3A, B). No labelling was found on cells other than those in the vascular system, i.e. on bronchioles, terminal bronchioles, type II alveolar epithelial cells and alveolar phagocytes. In the rat which had received an excess amount of unlabelled AM (5 nmol/kg), some silver grains were observed in some parts. However, their distribution was scattered on any structures of the lung (Fig. 3E).

In the kidney of the rat administrated ^{125}I -AM alone, silver grains were found in the proximal convoluted tubular cells (Fig. 4A, B). No grains were found in other parts, e.g. the pericapillary space, glomeruli, distal tubules, collecting tubules or medulla.

In the liver, a small number of silver grains was observed along the sinusoidal capillaries in the liver lobules (Fig. 4C, D). Most of the grains observed were on the Kupffer cells (Fig. 4D, arrows).

Electron microscopic autoradiography (EM-ARG)

EM-ARG in the lung showed, a large number of phenidon-developed silver grains on the endothelial cells of the alveolar capillaries (Fig. 5A) and pulmonary venules (Fig. 5B) in the rat at 2 min after administration of ^{125}I -AM alone. No silver grains were observed on the type I and II alveolar epithelial cells or alveolar phagocytes.

In the kidney, a few developed silver grains were observed on the apical side of the proximal convoluted tubules (data not shown).

In the liver, the grains were mainly observed on the Kupffer cells and along the sinusoidal capillaries by LM-ARG. However, due to the low number of phenidon-developed silver grains in these sites, it was not possible to evaluate the binding sites of ^{125}I -AM at the ultrastructural level.

IV. Discussion

Good fixation of the radiolabelled compounds is a prerequisite for autoradiographic studies. Proteins and peptides have been found to be more effectively fixed when tannic acid is present in the aldehyde fixative solution [8, 11]. In our *in vitro* test, we added 0.1% tannic acid, 2% formaldehyde and 0.5% glutaraldehyde to the ^{125}I -AM solution. ^{125}I -AM precipitated forming an insoluble complex with tannic acid and aldehydes, and 100% of the radioactivity was found in the precipitate. Good fixation of compound was confirmed in our autoradiography

Fig. 3. LM-ARG of the lung after administration of ^{125}I -AM without (A–D) and with an excess of unlabelled AM (E). Silver grains were localized on the endothelial cells of small arteries (25 μm in diameter), whereas scarce labelling was found on the smooth muscle (A). Silver grains were rare on the endothelial and smooth muscle cells of the large arteries (B). Some silver grains were also observed along the alveolar capillaries (C, arrowheads) and venules (C, arrow and D). Grains were very sparse (E) compared with the case of administration of ^{125}I -AM alone (A, C, D). A, B, E: $\times 500$, Bar = 20 μm , C, D: $\times 1200$, Bar = 10 μm .

Fig. 3

Fig. 4. LM-ARG of the kidney (A, B) and liver (C, D) after administration of ^{125}I -AM. Silver grains were localized on the apical side of the proximal convoluted tubular cells (A, B). The grains were located along the sinusoidal capillaries in the liver lobules (C) and on the Kupffer cells (D). Arrows indicate the Kupffer cells. A, C: $\times 500$, Bar = $20\ \mu\text{m}$, B, D: $\times 1200$, Bar = $10\ \mu\text{m}$.

study. Through dehydration and embedding process, 98.2% of the radioactivity was retained in the tissues, not being washed out by ethanol solutions or resin.

In this study, we investigated the binding sites of ^{125}I -human-AM in the rat tissue by whole-body, light microscopic (LM) and electron microscopic (EM) autoradiography (ARG). The highest radioactivity was found in the lung by whole-body-ARG. This high ac-

cumulation may reflect the specific binding of ^{125}I -AM. This finding is consistent with the results of a binding assay using a membrane fraction by Owji *et al.* [13], which showed that the ^{125}I -AM binding sites were most abundant in the lung. Because immunoreactive AM and a large amount of AM mRNA has been detected in the lung [14, 15], AM may act in an autocrine and/or paracrine manner in the lung. Physiologically, AM causes a decrease in

Fig. 5. EM-ARG of the lung after administration of ^{125}I -AM. Silver grains were densely localized on the capillary endothelium of the alveolar walls (A). Many silver grains were also observed on the endothelial cells of the pulmonary venules (A). Developed with phenidon developer. A: $\times 6800$, B: $\times 8800$, Bar = $2\ \mu\text{m}$.

Fig. 5

pulmonary vascular resistance [9, 19].

LM and EM-ARG showed that the major binding sites of AM in the rat lung were on the endothelial cells of small arteries, capillaries and venules. It has been suggested that AM-induced vasodilation may be mediated through elevation of cAMP in smooth muscle cells and through the release of vasodilators (e.g. endothelium-derived relaxing factor) from endothelial cells [16]. In the present study, the vascular smooth muscle cells along the entire pulmonary vascular system did not show any labeling. Addition of AM to the culture medium *in vitro* has been shown to increase intracellular cAMP in VSMC [1]. The reason for this apparent discrepancy with other reports is not clear at present. One possible explanation is that only a small number of AM receptors on the VSMC may be sufficient for vasodilation.

Although the binding of AM to large arteries was scarce, we observed many grains on the endothelium in the small arteries which were 20–50 μm in diameter (Fig. 3A). This is the terminal part of the arteries and is known to regulate blood flow. Our observations are consistent with the result of a recent pharmacological study by Shirai *et al.* [17]. They used an X-ray television system on the cat pulmonary vasculature and observed that AM-induced vasodilation was greater in the smaller arteries (100–500 μm) than in the larger arteries (500–1100 μm).

In the whole-body-ARG experiment, the next highest level of radioactivity was found in the renal cortex and liver. The simultaneous administration of a large amount of unlabelled AM did not abolish the binding of ^{125}I -AM to the renal cortex and liver. In the kidney, at the micro- and ultrastructural levels, the radioactivity was confined to the apical side of the proximal convoluted tubules, which showed that the radioactivity found in the renal cortex was not due to the binding to the AM-specific receptors, but probably due to glomerular filtration and reabsorption. Proximal tubules are known to nonspecifically absorb various kinds of proteins and peptides. In the liver, silver grains were observed along the sinusoidal capillaries in LM-ARG, suggesting that binding sites may be on Kupffer cells and/or sinusoidal endothelial cells. However, due to low radioactivity in the liver tissues, it was not possible to visualize the AM binding sites further in detail using EM-ARG technique. When unlabelled AM was administered simultaneously, ^{125}I -AM binding to the liver was not abolished, rather, radioactivity was higher than the rats which received ^{125}I -AM alone. We speculate the reason for this is as follows: Injected into tail vein, ^{125}I -AM flows through the right atrium and ventricle and into lung vasculature, where a large portion of the peptide may be trapped and only untrapped peptide may go into systemic circulation. When a large amount of unlabelled AM was administered with ^{125}I -AM, labelled peptide may escape entrapment in the lung, thus larger amount of ^{125}I -AM could reach the liver, kidney and other organs.

In the present study, we undertook to investigate major binding sites of ^{125}I -AM in the body and thus gain

an insight into the physiological role of this peptide. For this purpose, we chose three organs i.e. lung, kidney and liver, which have the most abundant binding sites. It would be of interest to investigate the localization of ^{125}I -AM binding sites in other organs, especially which have sinusoidal capillaries such as the spleen, bone marrow and adrenal glands. Further study will clarify detailed localization of specific binding sites in these organs.

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VI. References

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