Atrial Natriuretic Polypeptide in Bovine Adrenal Medulla

**Masashi Mukoyama, Kazuwa Nakao, Narito Morii, Shozo Shiono, Hiroshi Itoh, Akira Sugawara, Takayuki Yamada, Yoshihiko Saito, Hiroshi Arai, and Hiroo Imura**

**SUMMARY** Two radioimmunoassays for α-human atrial natriuretic polypeptide (α-hANP) with different specificities were used to study the tissue level and the nature of α-hANP-like immunoreactivity in the bovine adrenal gland. A considerable amount of α-hANP-like immunoreactivity was detected in the adrenal medulla (90.8 ± 21.1 and 90.0 ± 23.1 ng/g with the two radioimmunoassays), while no detectable amount (<1.0 ng/g) was present in the cortex. Gel permeation chromatographic analysis showed that ANP in the medulla is composed of two components of α-hANP-like immunoreactivity with high and low molecular weights in the approximate ratio of 2:1, eluting at the elution positions of γ-hANP and α-hANP, respectively. Reverse-phase high performance liquid chromatographic analysis revealed that α-hANP-like immunoreactivity with a low molecular weight in the medulla consists of two major components, which comigrate with synthetic α-hANP(5-28) and α-hANP. When cultured bovine adrenal chromaffin cells were incubated in the presence of nicotine (10^-5 M), α-hANP-like immunoreactivity was released into the medium concomitantly with catecholamines from chromaffin cells. These findings indicate that a discrete ANP system is present in the adrenal medulla and that ANP is cosecreted with catecholamines from chromaffin cells, suggesting the possible involvement of ANP in the adrenomedullary function. (Hypertension 11: 692-696, 1988)

**KEY WORDS** • atrial natriuretic factor • radioimmunoassay • catecholamine • nicotine • chromaffin cell • adrenomedullary function

ATRIAL natriuretic polypeptides (ANPs), also known as atrial natriuretic factor (ANF), a family of vasoactive peptides with potent diuretic, natriuretic, and vasorelaxant properties, have been isolated from human and rat atrial tissues and implicated in the control of water and electrolyte balance and blood pressure. Using a radioimmunoassay (RIA) for α-ANP which recognizes α-human ANP (α-hANP or human ANF-[99-126]) and α-rat ANP (α-rANP or rat ANF-[99-126]) equally, we have demonstrated that ANP is secreted from the heart and circulates in the body as a hormone. Furthermore, widespread distribution of α-ANP-like immunoreactivity (LI) has been demonstrated in extraatrial tissues including the brain, spinal cord and autonomic ganglia, kidney, and lung. The chromatographic analyses revealed that the molecular form of ANP in the rat central nervous system is different from that in the heart; the predominant form of ANP in the brain and spinal cord is a low molecular weight form, major components of which are α-rANP(4-28) (rat ANF-[102-126]) and α-rANP(5-28) (rat ANF-[103-126]). We also reported the effects of ANP on the central nervous system, including inhibitory actions on water and salt intake, pressor response to angiotensin II, the release of vasopressin and adrenocorticotropic hormone, and on the central dopaminergic system. These findings suggest that ANP plays a role in the central control of fluid homeostasis and blood pressure as a neuropeptide. In addition to the possible role of ANP in the central...
nervous system, there is growing evidence that ANP is implicated in the peripheral sympathetic nervous system. 17 Furthermore, immunohistochemical studies have shown the existence of ANP in the adrenal medulla. 18, 19 However, little is known about ANP in the adrenal medulla. In this study, we investigated the presence and the nature of ANP in the bovine adrenal medulla and the possible release of ANP from adrenal chromaffin cells.

Materials and Methods

Tissue and Extraction

Bovine adrenal glands were obtained from a local slaughterhouse and chilled on ice immediately after removal from the animals. Within an hour, the medulla and cortex were dissected out, immediately boiled for 5 minutes in 10 volumes (2-5 ml) of 0.1 M acetic acid, and then homogenized with a polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland) for 60 seconds, as previously reported. 5, 8, 10-13 The homogenate was centrifuged at 18,000 g for 30 minutes at 4°C, and the supernatant was stored at -20°C until assay. The adrenal extract was applied to a Sep-Pak C18 cartridge (Waters Associates, Milford, MA, USA), and adsorbed peptides were eluted with 2 ml of 60% acetonitrile in 0.1% trifluoroacetic acid. The eluate was lyophilized and subjected to high performance gel permeation chromatography (HP-GPC) and reverse-phase high performance liquid chromatography (RP-HPLC).

Peptides

Synthetic α-hANP, α-hANP-(4-28) (human ANF-[102-126]), α-hANP-(5-27) (human ANF-[103-125]), α-hANP-(5-28) (human ANF-[103-126]) and α-hANP-(7-28) (human ANF-[105-126]) were purchased from Peptide Institute, Osaka, Japan. γ-Human ANP (γ-hANP or human ANF-[1-126]) was kindly donated by Professor Matsuo, Miyazaki Medical College, Miyazaki, Japan.

Radioimmunoassay

α-hANP-LI in the extract was measured using two RIAs for α-hANP with different specificities. The antiserum used in the first RIA was raised in rabbits against the C-terminal fragment of α-hANP, α-ANP-(17-28) (ANF-[115-126]). 5 The RIA recognizes α-hANP, α-hANP-(4-28), α-hANP-(5-27), α-hANP-(5-28), α-hANP-(7-28) and γ-hANP equally and shows a cross-reactivity of 11.0% with α-hANP-(5-27) on a molar basis. The antiserum in the second RIA was kindly donated by Mitsubishi Petrochemical, Tokyo, Japan. The RIA recognizes the ring structure of α-hANP including [Met12] and shows equimolar cross-reactivities with α-hANP-(4-28), α-hANP-(5-27), α-hANP-(5-28), α-hANP-(7-28) and γ-hANP.

Catecholamine Assay

The levels of epinephrine and norepinephrine in the culture medium of chromaffin cells were measured using HPLC with electrochemical detection, as described elsewhere. 16

High Performance Gel Permeation Chromatography

HP-GPC was performed on a TSK-GEL G2000 SW column (7.5 × 600 mm; Toyo Soda, Tokyo, Japan) as previously described. 5, 8, 10-13 The α-hANP-LI level in the eluate was assayed with the two RIAs just described.

Reverse-Phase HPLC

RP-HPLC was performed on a TSK-GEL octadecylsilane (ODS) 120T column (4.6 × 75 mm; Toyo Soda). 10, 13 The α-hANP-LI level in the eluate was determined by the two RIAs.

Release Study with Cultured Chromaffin Cells

A primary culture of bovine adrenal chromaffin cells was prepared by retrograde perfusion with collagenase according to the method described by Kumakura et al. 20 After 2 days of maintenance in culture, approximately 5 × 10⁶ cells were used for the following release study. The chromaffin cells were washed twice with Krebs-Ringer bicarbonate-glucose buffer (pH 7.4) containing 0.5% bovine serum albumin and dispersed in the same solution at 37°C. After the 10-minute preincubation period, the medium was replaced and the cells were incubated in the presence of 10⁻³ M nicotine for 10 minutes. The α-hANP-LI level in the medium was measured using RIAs after extraction with a Sep-Pak C₁₈ cartridge.

Results

ANP Levels in the Adrenal Gland

Serial dilutions of extracts from the bovine adrenal medulla gave competition curves parallel to the standard curve of α-hANP in each RIA. Extracts from the adrenal cortex, however, caused no displacement of bound ¹²⁵I-α-hANP in either RIA. Table 1 gives the concentrations of α-hANP-LI in the bovine adrenal medulla and cortex. A highly significant correlation was observed between values of α-hANP-LI determined with the two RIAs (r = 0.992, p < 0.001).

HP-GPC Analysis

Figure 1 shows the HP-GPC profiles of α-hANP-LI in the bovine adrenal medulla assayed with two RIAs. As shown in Figure 1A, α-hANP-LI in the adrenal medulla was composed of two peaks. The major peak

<table>
<thead>
<tr>
<th>Tissue</th>
<th>α-hANP-LI (ng/g wet tissue)</th>
<th>RIA for C-terminus</th>
<th>RIA for midportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal medulla (n = 8)</td>
<td>90.8 ± 21.1</td>
<td>90.0 ± 23.1</td>
<td></td>
</tr>
<tr>
<td>Adrenal cortex (n = 8)</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td></td>
</tr>
</tbody>
</table>

Medulla values are expressed as the mean ± SE. LI = like immunoreactivity.
eluted at the position with an approximate molecular weight of 13,000 and appeared to correspond to γ-ANP. The minor peak, with a low molecular weight, eluted at the position corresponding to that of synthetic α-hANP. The ratio of α-hANP-LI between the two peaks (high:low) was approximately 2:1. The results obtained with the second RIA were essentially identical (see Figure 1B). The recoveries of α-hANP-LI in HP-GPC were 107 and 87% in the first and the second RIA, respectively.

**RP-HPLC Analysis**

To further characterize α-hANP-LI in the adrenal medulla, RP-HPLC coupled with RIA was performed. As shown in Figure 1C, α-hANP-LI with a low molecular weight comprised two major peaks eluting in the vicinity of the position of synthetic α-hANP. The elution positions of these two peaks corresponded to those of α-hANP-(5-28) and α-hANP. The peak corresponding to α-hANP-(5-28) was larger than that corresponding to α-hANP. Characters of these two components were verified by confirming their comigration with synthetic peptides when mixed with the extract and subjected to RP-HPLC. In addition, a much higher peak was observed at the elution position corresponding to γ-ANP. RP-HPLC coupled with the second RIA gave essentially the same pattern as that shown in Figure 1C.
Corelease of ANP and Catecholamines from Chromaffin Cells

Table 2 shows the results of the release study with cultured adrenal chromaffin cells. α-hANP-LI was released concomitantly with catecholamines from adrenomedullary chromaffin cells by nicotine.

Discussion

The present study demonstrates that ANP, originally isolated as a cardiac hormone and subsequently shown to exist in the central nervous system as a neuropeptide,1-10, 13 is also present in the bovine adrenal medulla. The tissue level of ANP in the bovine adrenal medulla was higher than that in the rat brain6 or spinal cord10 and comparable to that in the human or rat ventricle.21, 22 The two different RIAs for α-hANP used in this study gave virtually identical results as to tissue levels and chromatographic patterns of α-hANP-LI in the adrenal medulla. This finding verifies the validity of the assay systems used in the present study. The extraction method used in this study is the same as that used by Kangawa and Matsuo2 when they isolated α-hANP from human atrial tissues and is known to minimize the nonspecific proteolysis producing artifacts.

Using the HP-GPC and RP-HPLC coupled with RIA, we previously reported that ANP stored in the rat heart is almost exclusively a high molecular weight form of ANP, γ-rANP, whereas the major components of ANP in the rat central nervous system are low molecular weight forms, α-rANP-(4-28) and α-rANP-(5-28).8-10, 13 Thus, we proposed that ANP in neurons is generated by a different posttranslational processing from that in cardiocytes.9 In the current study, HP-GPC analysis revealed that α-hANP-LI in the bovine adrenal medulla consists of two components with high and low molecular weights corresponding to γ-ANP and α-ANP, respectively, in the approximate ratio of 2:1. This appears to be an intermediate pattern between those found in the heart and in the brain. Furthermore, in the RP-HPLC study, α-hANP-LI with a low molecular weight in the medulla was composed of two major components that comigrated with synthetic α-hANP-(5-28) and α-hANP. Since the amino acid sequence of bovine α-ANP is identical with that of α-hANP,23 it seems very likely that these two components in RP-HPLC are α-hANP-(5-28) and α-hANP, respectively. The presence of the precursor, γ-ANP, indicates the possible biosynthesis of ANP in the adrenal medulla. Therefore, the processing mechanism of ANP in the bovine adrenal medulla appears to differ from that in the heart or in the central nervous system and, interestingly, shows an apparently intermediate pattern between those of the two systems. This again raises the possibility of a tissue-specific processing mechanism of a common ANP precursor. However, the possibility that the difference in the molecular form of ANP may be due to the species difference rather than the tissue difference cannot be ruled out, and the definitive interpretation of the result must await further clarification.

The present study also showed that ANP is released into the medium together with catecholamines from chromaffin cells by nicotine administration. Since nicotine stimulates secretion of catecholamines stored in chromaffin granules of adrenomedullary cells, it is likely that ANP coreleased with catecholamines by nicotine is also stored in chromaffin granules, like neuropeptides such as opioid peptides. During the preparation of this article, the presence of ANP in the bovine adrenal chromaffin granules was reported by Ong et al.24 using radioceptor assay and amino acid analysis. They isolated ANF-(99-126) and the precursor, ANF-(1-126), from the granules. They detected ANP activity by radioceptor assay, but the specificity of the assay was not clear, especially in binding with the precursor, γ-ANP, and α-ANP-(5-28). We used RIAs in this study, and our RIAs can detect α-ANP, α-ANP-(5-28) and γ-ANP equally. Therefore, the difference between the results could be explained in part by different detection methods.

The role of ANP in the adrenal medulla is not clear at present. Accumulated evidence suggests an inhibitory role of ANP in the sympathetic nervous system.17 Therefore, ANP in the adrenal medulla may participate in the inhibitory control of catecholamine secretion from adrenomedullary chromaffin cells. In conclusion, these results indicate that a discrete ANP system is present in the adrenal medulla, suggesting the possible involvement of ANP in the adrenomedullary function.

Acknowledgments

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References

1. Flynn TG, de Bold ML, de Bold AJ. The amino acid sequence of an atrial peptide with potent diuretic and natriuretic properties. Biochem Biophys Res Commun 1983;117:589-595
7. Sugawara A, Nakao K, Morii N, et al. Significance of α-

Table 2. Nicotine-Induced Corelease of α-hANP-Like Immunoreactivity with Catecholamines from Cultured Bovine Adrenomedullary Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Epinephrine (µg)</th>
<th>Noradrenaline (µg)</th>
<th>α-hANP-LI (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.60 ± 0.03</td>
<td>0.34 ± 0.02</td>
<td>&lt;3.0</td>
</tr>
<tr>
<td>10^-3 M nicotine</td>
<td>2.2 ± 0.11</td>
<td>2.3 ± 0.12</td>
<td>15 ± 0.50</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SE for four separate experiments and represent the amount released per 10^6 cells for 10 minutes. LI = like immunoreactivity.
human atrial natriuretic polypeptide as a hormone in humans. Hypertension 1986;8(suppl I):1-151–1-155

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