Distribution of Immunoreactive Atrial and Brain Natriuretic Peptides in the Heart of the Chicken, Quail, Snake and Frog

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Abstract: The distribution of atrial natriuretic peptide (ANP)- and brain natriuretic peptide (BNP)-granules was examined immunohistochemically and ultrastructurally in the hearts of the chicken, Japanese quail, Japanese rat snake and bullfrog. Moreover, natriuretic peptide (NP)-granules in the cardiocytes were analyzed by ultrastructural morphometry. Immunohistochemically, ANP-immunoreactivity (IR) was not detected in any cardiocytes, but BNP-IR was detectable in most atrial and ventricular cardiocytes of both chicken and quail. In the snake, ANP-IR was seen in most atrial and ventricular cardiocytes, which showed traces and negative in BNP-IR, respectively. Both ANP- and BNP-IR were detected in the atrial and ventricular cardiocytes in the frog. Ultrastructurally, most of NP-granules were found in the perinuclear region in the chicken, quail and snake atrium, but the frog atrial cardiocytes had granules generally dispersing widely in the cell. By ultrastructural morphometry, the number of granules in the atrial cardiocyte was greatest in the frog, followed by the snake, and chicken or quail, in this order. The diameter of granules in the atrial cardiocyte was largest in the snake and reduced via the frog to the chicken or quail. In the ventricular cardiocytes of all species, the number and size of granules were significantly less than that in the atrial ones. These results indicated that the hearts of the chicken and quail contain only BNP, and that there are two different natriuretic peptides, ANP and BNP, in the snake and frog hearts.

Key words: heart, immunohistochemistry, natriuretic peptide, non-mammals, ultrastructural morphometry

Introduction

It is well known that atrial cardiocytes in mammals are endocrine cells which secrete atrial (A-type) natriuretic peptide (ANP) [6, 8] which has diuretic, natriuretic and vasodilatory properties and exerts an inhibitory action on aldosterone, cortisol, arginine vasopressin and renin release, and ANP is a peptide

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hormone believed to be involved in blood pressure and volume homeostasis [36]. Recently, many immunohistochemical studies have been made on ANP in vertebrate hearts. By means of morphometry, the size and number of atrial secretory granules were examined in various mammalian species by Cantin et al. [5], Jamieson and Palade [11], Mifune et al. [15, 16, 19, 20], and Tomisawa [31], but no report has been presented on the size and number of atrial secretory granules in non-mammalian hearts.

On the other hand, a natriuretic peptide, similar to ANP in primary structure, has been isolated from the porcine brain and designated as brain (B-type) natriuretic peptide (BNP) [29]. BNP, which has similar physiological actions to ANP, also occurs in the hearts of the pig [1], rat [2], dog [27] and man [12], and the species differences in the primary structure and distribution of BNP in mammalian tissues are well-known. In non-mammalian hearts, a few studies of BNP have been conducted especially in chicken [3, 32], urodele [13] and fish [7], but no species differences in the amino acid sequence and immunoreactivity of BNP in non-mammalian hearts have been demonstrated.

As part of a series of comparative morphological studies on the secretory granules in the cardiocytes, the present study was designed to describe immunohistochemically, ultrastructurally and morphometrically the secretory granules in the chicken, quail, snake and frog hearts. In addition, these findings were compared with those of atrial secretory granules in the mammalian heart which we reported previously. The aim of this study was also to determine whether porcine BNP can be detected in the heart of these non-mammals, and if so, whether or not there are species differences in porcine BNP immunoreactivity in these hearts.

Materials and Methods

Animals: Five male animals were obtained from each group of 6-month-old chickens (White-Leghorn), 3-month-old Japanese quails (Coturnix coturnix japonica), Japanese rat snakes (Elaphe climacophora, 100–140 cm in length) and bull-frogs (Rana catesbeiana, 14–17 cm in length), which were collected in summer (June–August), and used as materials in this study. Atrial and ventricular tissues were removed from these animals under Nembutal anesthesia.

Immunohistochemistry: The atrial and ventricular tissue blocks were fixed in Zamboni’s solution for 24 hr at 4°C. After washing with 0.15 M phosphate-buffered saline (PBS) at pH 7.3, they were dehydrated, embedded in paraffin and sectioned at 5 μm. Prior to immunohistochemical staining, the deparaffinized sections were incubated in absolute methanol containing 3% H2O2 for 20 min. Immunohistochemical staining was performed according to the modified avidin-biotin peroxidase complex (ABC) technique described in our previous report [18]. Following incubation in normal swine serum (for primary antibody raised in rabbit) or normal rabbit serum (for monoclonal antibody), sections were incubated with primary antibody overnight at 4°C. For ANP immunohistochemical study, rabbit antiserum against synthesized human ANP/cardiodilatin 99–126 [Code: NAW160] [25] was used as the primary antibody, diluted 1:1,000 with PBS containing 0.02% Triton X 100. Additionally, for a BNP immunohistochemical study, monoclonal antibody against synthesized porcine BNP 1-32 [Code: KY-BNP-II] [10] was used as the primary antibody, diluted 1:1,000–1:2,000 with PBS containing 0.02% Triton X 100. This monoclonal antibody was kindly donated by Prof. Nakao of Kyoto University, Japan. As the secondary antibody, biotinylated swine anti-rabbit immunoglobulin (for primary antibody raised in rabbit) (1:500; DAKO, Denmark) or biotinylated rabbit anti-mouse immunoglobulin (for monoclonal antibody) (1:300; DAKO, Denmark) was used.

Electron microscopy: The atrial and ventricular tissues were fixed in 2% paraformaldehyde – 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 and post-fixed in 1% osmium tetroxide in the same buffer. They were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate, and examined with a JEM-2000 EX electron microscope.

Ultrastructural morphometry: The number and diameter of the atrial secretory granules of the atrial and ventricular cardiocytes were measured according to our previous methods [15, 16]. In each species, the means
(± SD) of counts were calculated in 50 photographs from 5 animals with the measurements of at least 300 granules in total and statistically analyzed by Student's t-test.

Results

Immunohistochemistry

The primary antibodies against ANP and BNP, preabsorbed with synthetic human or rat ANP and porcine BNP, respectively, showed no detection of immunoreaction.

Atrium: ANP-immunoreactivity (IR) was not detected in any cytoplasm of the atrial cardiocytes of either chicken or quail, but BNP-IR was detectable in most atrial cardiocytes of these fowls (Fig. 1A, B). In the snake, ANP-IR was seen in most atrial cardiocytes, which showed traces of BNP-IR (Fig. 1C, D). In the frog, both ANP- and BNP-IR were detected in the atrial cardiocytes. Further, when consecutive sections were compared (Fig. 1E versus 1F), ANP- and BNP-IR appeared to be localized in the same cardiocytes of the frog, and BNP-IR was obviously weaker than ANP-IR. The immunoreaction for ANP was most intense in the frog atrium, and was moderate in the snake atrium. Antibody against BNP produced a weak reaction in the atria of the chicken, quail and frog (Fig. 1). These reaction deposits for ANP and / or BNP were demonstrated in the perinuclear regions of cardiocytes in all species.

Ventricle: ANP- and BNP-IR in the ventricular cardiocytes in the chicken, quail (Fig. 2A vs 2B) and frog (Fig. 2C vs 2D) were similar in intensity to that in the atrial ones in these species. In the snake, ANP-IR was seen in most ventricular cardiocytes, but no BNP-IR was observed in the cells (Fig. 2E vs 2F). In all the species examined, ANP- and/or BNP-IR in the ventricular cardiocytes were weaker than those in the atrial ones.

Ultrastructure

In the atrium and ventricle of all species, the cardiocyte contained a centrally located nucleus, numerous mitochondria, myofibrils, a few rough endoplasmic reticula, Golgi apparatus and electron-dense granules (Fig. 3). In the chicken, quail and snake atrium, most of the granules were found at the poles of the elongated nucleus (Fig. 3A, B), but the frog atrial cardiocytes generally had granules widely dispersed in the cell (Fig. 3C). In the atrium, the granules in the chicken and quail (Fig. 3A) were smaller in size and fewer in number than those in the snake (Fig. 3B) and frog (Fig. 3C). In addition, the Golgi apparatus of atrial cardiocytes was well developed in the snake and frog, but poorly-developed in the chicken and quail. In all species, the granules in the ventricular cardiocytes were smaller in size and fewer in number than those in atrial ones (Fig. 3A vs 3D; 3B vs 3E; 3C vs 3F), and most of the granules were observed in the perinuclear region associated with the Golgi apparatus.

Ultrastructural morphology

The number and diameter of granules in the atrial and ventricular cardiocytes were analyzed as shown in Table 1. In the atrial cardiocytes, the number of granules was significantly greater in the frog than in the snake, chicken or quail (p<0.01), and significantly greater in the snake than in the chicken and quail (p<0.01). There was no significant difference between the chicken and quail in the number of granules in the atrial cardiocytes. In the ventricular cardiocytes of all species, the number of granules was significantly fewer than that in the atrial ones (p<0.01). The number of granules in the ventricular cardiocytes was significantly greater in the frog than in other species (p<0.01), but there was no significant difference between the chicken, quail and snake in the number of granules in the ventricular cardiocytes. On the other hand, the diameter of granules in the atrial and ventricular cardiocytes was significantly larger in the snake than in the frog, chicken and quail (p<0.01), and significantly larger in the frog than in the chicken and quail (p<0.01). There was no significant difference between the chicken and quail in the diameter of granules in the atrial and ventricular cardiocytes. In the ventricular cardiocytes of all species, the diameter of the granules was significantly smaller than that in the atrial ones (p<0.01).

Discussion

Immunohistochemistry

As the primary structures of snake and frog ANP, and quail, snake and frog BNP are unknown, this immunohistochemistry was performed by using antiserum
raised against human ANP (1–28) in rabbits and monoclonal antibody against synthesized porcine BNP (1–26). In a preliminary experiment, this anti-human ANP antiserum had a 100% cross-reactivity with human and rat ANP (1–28), but did not cross-react with human, rat or porcine BNP. This monoclonal antibody against porcine BNP had 100% cross-reactivity with porcine BNP-26, 55% cross-reactivity with porcine BNP-32, and less than 0.001% cross-reactivity with human ANP-28 and rat ANP-28 [10]. In preabsorption controls and cross-reactivity tests, preabsorption of this anti-ANP antiserum with synthetic human, rat and porcine BNP resulted in no detectable changes in immunostaining, as compared with results obtained with the untreated

Fig. 1. Immunohistochemical staining of ANP (left side: A, C, E) and BNP (right side: B, D, F) in atrium in the quail (A, B), snake (C, D) and frog (E, F). All magnifications are ×314. In the quail (A, B), ANP-IR is not detected in any cytoplasm, but BNP-IR is detectable in most cardiocytes. In the snake (C, D), ANP-IR is seen in most cardiocytes, with traces in BNP-IR. In the frog (E, F), both ANP- and BNP-IR are detected in cardiocytes. The immunoreaction for ANP is most intense in the frog atrium (E), and is moderate in the snake atrium (C). Antibody against BNP produces a weak reaction in the quail (B) and frog (F). These reaction deposits for ANP and/or BNP are demonstrated in the perinuclear regions of cardiocytes in all species (arrowheads).
anti-human ANP antiserum. Furthermore, the immunoreactivity obtained from the antibody against porcine BNP used in this study was preabsorbed by porcine BNP, but was unaffected by incubation with human and rat ANP, and BNP. We therefore consider that the detected peptide as 'ANP-like' or 'BNP-like' is referred to the immunoreaction obtained as ANP-immunoreactive and BNP-immunoreactive, respectively.

This study immunohistochemically demonstrated the distribution of immunoreactive atrial and brain natriuretic peptides in the hearts of chicken, quail, snake and frog. ANP-IR was not detected in any cytoplasm of the atrial or ventricular cardiocytes of chicken and quail, but BNP-IR was detectable in most of these cardiocytes. Toshimori et al. [32] reported that the chicken ANP was considered to belong to the BNP-type rather than the ANP-type. And Akizuki et al. [3] has isolated from chicken heart a novel natriuretic pep-

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**Fig. 2.** Immunohistochemical staining of ANP (left side: A, C, E) and BNP (right side: B, D, F) in ventricle in the quail (A, B), frog (C, D) and snake (E, F). All magnifications are × 314. In the quail (A, B), ANP-IR is not detected in any cytoplasm, but BNP-IR is detectable in most cardiocytes. In the frog (C, D), both ANP- and BNP-IR are detected in cardiocytes. In the snake (E, F), ANP-IR is seen in most cardiocytes, but no BNP-IR is observed in cardiocytes. In all the species examined, ANP- and/or BNP-IR in the cardiocytes are weaker than those in atrial cardiocytes.
Fig. 3. Electron micrographs of atrial (left side; A, B, C) and ventricular (right side; D, E, F) cardiocytes in the quail (A, D), snake (B, E) and frog (C, F). Each bar represents 1 μm. Nucleus (N), NP-granules (arrowheads). In the atrium, granules in the quail (A) are smaller in size and fewer in number than those in the snake (B) and frog (C). In the quail and snake atrium, most of the granules are found at the poles of the elongated nucleus, but the frog atrial cardiocytes generally have granules widely dispersed in the cell. In all the species examined, the granules of ventricular cardiocytes are smaller in size and fewer in number than those of atrial ones (A vs D, B vs E, C vs F).
Table 1. Comparison of the number and diameter of natriuretic peptide granules in the atrial and ventricular cardiocytes (means ± SD)

<table>
<thead>
<tr>
<th>Species</th>
<th>Number</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Atrium</td>
<td>Ventricle</td>
</tr>
<tr>
<td>Chicken</td>
<td>7.2 ± 1.4</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>J. quail</td>
<td>10.3 ± 1.6</td>
<td>4.6 ± 1.3</td>
</tr>
<tr>
<td>Snake</td>
<td>30.4 ± 8.6*</td>
<td>4.1 ± 1.4</td>
</tr>
<tr>
<td>B. frog</td>
<td>119.4 ± 10.9**</td>
<td>13.5 ± 2.6**</td>
</tr>
</tbody>
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* Significantly different from chicken, quail and frog (p<0.01). ** Significantly different from chicken, quail and snake (p<0.01). * Significantly different from ventricle (p<0.01).

tide of 29 amino acid residues, which was highly homologous to porcine BNP at the C-terminus. Our immunohistochemical results agree with theirs, and suggest that the quail heart also contains only BNP. To the best of our knowledge, the primary structures of snake ANP and BNP, and frog BNP are unknown. In this study, ANP-IR was seen in both atrial and ventricular cardiocytes in the snake and frog, but the atrial and ventricular cardiocytes showed traces and negative, respectively, in BNP-IR in the snake, and showed positive in BNP-IR in the frog. The primary structures of the snake and frog ANP are therefore homologous to man, rat and other mammalian ANPs, and our results indicate that there are two different peptides similar to mammalian ANP and porcine BNP in the frog heart. It is also possible that the structure of the snake BNP is different from the porcine BNP-type.

Ultrastructure and ultrastructural morphometry

Although the ultrastructural evaluation of atrial and ventricular cardiocytes showed electron-dense granules in all species examined in this study, the number and size of granules in the cell differed among species. The numerical differences in natriuretic peptide (NP) granules paralleled the different intensity of ANP-IR or BNP-IR in the cardiocytes among these species. On the other hand, the distribution pattern of granules in the atrial and ventricular cardiocytes was common to the chicken, quail and snake in that the granules were more frequently observed in the perinuclear region than between myofibrillar bundles. These cellular distributions of NP-granules were consistent with those in the mammalian cardiocytes [15, 16, 19, 20]. In the frog atrium, NP-granules were widely localized in the cytoplasm except in the perinuclear region in the cell. This distribution pattern of granules was also reported in the newt ventricle [13] and some kinds of fish heart [34]. It is possible that such a distribution pattern of the granules is a characteristic of amphibia and fishes. The different distribution pattern of the granules in the frog atrium from the mammalian one may be caused by the difference in the secretory pathway in the cell in the frog and mammalian secretion-type.

In ultrastructural morphometry, there were numerical differences in NP-granules in non-mammalian species. In general, numerical changes in NP-granules are observed under various physiological conditions. The number of NP-granules in the cell increases during dehydration [9], but decreases after Na loading [26]. Biochemically, the plasma ANP concentration and the cellular ANP mRNA levels decrease during dehydration [22, 30]. Conversely, both the plasma ANP and ANP mRNA levels increase after Na loading [22, 30]. These findings suggest that numerical changes in NP-granules are closely associated with their synthesis and secretion in the cardiocytes, and that the synthetic and secretory ability is enhanced in the cell with fewer granules. The numerical differences in NP-granules in non-mammalian species are suggested to be associated with differences in the synthetic and secretory ability in these species. It is possible that the synthetic and secretory ability is enhanced in the species with fewer granules in the cardiocytes. But the Golgi apparatus of the cardiocytes in the species with fewer granules and weaker ANP- or BNP-IR were poorly developed in this study. Relatively low production of the peptide and / or a rapid secretion, with a consequent lower storage of the peptide in the species, may be the reason.
The size of NP-granules varies according to the physiological state and is small in the presence of water depletion [9]. Biochemically, the plasma ANP concentration and cellular ANP mRNA level decrease during dehydration [30], and NP-granules become smaller during rapid synthesis, especially in spontaneously hypertensive rats [28]. In our previous reports, the enhancement of ANP synthesis in the cells reduced the granule size [17], or NP-granules became smaller with a concomitant decrease in the levels of atrial ANP mRNA and plasma ANP in the course the down-regulation [14]. These findings suggest no relationship between the granule size and the ability of ANP synthesis and secretion. In this study, there were differences in granule size among non-mammalian species. The dimensional differences in NP-granules in vertebrate are possibly related to other factors exclusive of the different ability for cellular ANP synthesis and secretion. Among various mammalian species in our previous reports [15, 16, 19, 20], the fewer number of granules the cardiocyte had, the smaller the granule size became. In this study, such an appearance was common to non-mammalian species, suggesting that the number of granules is possibly related to the granule size. The granule size is therefore possibly determined by the number of granules in the cardiocytes in the species.

In all the species examined, the ventricular cardiocytes contained fewer granules than atrial ones. A similar appearance has been reported in non-mammalian hearts, especially in the chicken [32], frog [23, 24] and fish [34, 35]. Further, in this study, ANP- and/or BNP-IR were observed in all working myocytes of non-mammalian ventricles. In general, NP-granules are only observed in the conduction system or Purkinje fiber in the mammalian ventricle, indicating that ANP plays a role as an impulse conductor in the conduction cells [4, 21, 33]. The ratio of peptide content in the ventricular tissue to that in atrial tissue is therefore larger in non-mammalian hearts than in mammalian ones, and may indicate a more important ventricular endocrine role in non-mammals than in mammals. ANP- and/or BNP-IR with fewer granules in working myocytes of the ventricle in non-mammalian species, is considered to reflect a high differentiation of the cardiocytes into cells having a much more endocrine function than mammalian ventricular cardiocytes, and, concomitantly, a relatively poorly endocrine function compared to atrium. It is possible that NP in non-mammalian ventricular endocrine cells plays a different role from ANP as an impulse conductor in mammalian conduction cells.

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References


