

## Different A-Type Natriuretic Peptide Level in Five Strains of Mice

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**ABSTRACT.** Atrial (A-type) natriuretic peptide (ANP) is vasodilative hormone involved in the regulation of blood pressure and volume homeostasis. In this study, we examined the differences of the auricular and plasma ANP distribution by immunohistochemistry, ultrastructural morphometry, and radioimmunoassay in five strains of mice. The ANP-immunoreactivities of the auricle were most intense in ICR, and moderate in C57BL/6J and C3H/HeN, and weakest in BALB/cA and DBA/2Cr. The number of ANP-granules was greatest in ICR followed by C57BL, C3H or BALB/c, and DBA/2 mice, in this order. The auricular ANP content was highest in ICR, but the plasma ANP concentration was comparable in all strains. The present study demonstrates that there are differences in the ANP circulating system between five strains.

**KEY WORDS:** A-type natriuretic peptide, cardiocyte, immunohistochemistry, mouse strain, ultrastructural morphometry.

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A-type (atrial) natriuretic peptide (ANP) is a circulating hormone with a wide range of biological effects, including natriuresis, diuresis and vasodilation, and it plays an important role in the regulation of blood pressure and volume homeostasis [7, 13, 15]. This peptide hormone is mainly produced in the cardiac atria, where it is stored within secretory granules as a large-sized precursor (proANP) [1, 3, 9–11].

Although most ANP studies have examined the biochemical and biophysical characteristics of this hormone, there have been few reports investigating the variations in the ANP-granule distribution in the hearts of various mammals by immunohistochemical or ultrastructural methods, since the electron-dense granules in the various mammalian atria have been reported as “specific atrial granules” [5]. Therefore, it is noteworthy to elucidate the morphological characteristics of the ANP-granules in many mammals by providing immunohistological or ultrastructural fragmentary information [2, 5].

Recently, many strains of mice have become available as a useful tool for biological research, and it is important to know precise and accurate information about each strain of interest. Regarding ANP, however, such strain differences have not been reported. In this context, the present study was designed to describe the immunohistochemical, ultrastructural and morphometrical differences

in the ANP-granules in five strains of mice, including four inbred strains and one outbred strain, together with the measurement of their plasma and auricular ANP levels.

**Animals:** Male inbred mice; C57BL/6J (C57BL), C3H/HeN (C3H), BALB/cA (BALB/c), and outbred mice; Jcl:ICR (ICR), purchased from CLEA Japan, Inc., (Osaka, Japan), and male inbred mice; DBA/2CrSlc (DBA/2) purchased from Japan SLC, Inc., (Hamamatsu, Japan), were used in this study (five animals and 12-week-old in each strain). All animals were kept in automatically controlled rooms (temperature: 24 ± 1°C; humidity: 50–60%; automatic lighting: 7:00 a.m. to 7:00 p.m.) and fed with a pellet diet CE-2 (CLEA Japan, Inc.) and water *ad libitum*. These animals were sacrificed under sodium pentobarbital anesthesia (50 mg/kg, i. p.) and their hearts and blood were removed. All experiments were undertaken in accordance with the *Guideline for Animal Experimentation*, Kurume University.

**Immunohistochemistry:** The right auricular tissue blocks were fixed in Zamboni’s solution for 24 hr at 4°C. Immunohistochemical staining was performed according to the modified avidin-biotin-peroxidase complex (ABC) technique, described previously [8]. Following incubation in normal swine serum, sections were incubated with primary antibody overnight at 4°C. In this immunohistochemical study, rabbit antiserum against synthesized human ANP 99–126 (Code: NAW160) [14] was used as the primary antibody diluted 1:1,000 with PBS containing 0.02% Triton X 100.

**Electron microscopy:** The right auricular tissues were fixed in 2% paraformaldehyde–2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 and post-fixed in 1% os-

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Table 1. ANP levels and blood pressure in five strains of mice

	ICR	C57BL/6J	C3H/HeN	BALB/cA	DBA/2Cr
Number of Auricular ANP-granules (count)	126.7 ± 9.8 <sup>a)</sup>	76.4 ± 19.6 <sup>b)</sup>	52.8 ± 19.2 <sup>c)</sup>	46.7 ± 17.1 <sup>c)</sup>	34.9 ± 16.9
Auricular ANP concentration (µg/g wet tissue)	101.5 ± 23.1 <sup>d)</sup>	57.2 ± 9.9	47.4 ± 4.4	57.1 ± 11.1	44.1 ± 9.4
Plasma ANP concentration (pg/ml)	177.8 ± 16.3	184.7 ± 12.6	182.5 ± 15.3	194.0 ± 22.6	186.6 ± 15.5
Systolic blood pressure (mmHg)	114.4 ± 9.5	108.6 ± 7.2	111.3 ± 6.8	116.9 ± 8.5	110.4 ± 5.3

Values are mean ± SD. Significant difference vs. C57BL/6J, C3H/HeN, BALB/cA, DBA/2Cr (a)  $P < 0.001$ , d)  $P < 0.01$ ). b) Significant difference vs. C3H/HeN, BALB/cA, DBA/2Cr ( $P < 0.01$ ). c) Significant difference vs. DBA/2Cr ( $P < 0.05$ ).

mium 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 of secretory granules of the right auricular cardiocytes was measured according to our previous report [12]. Thin sections were viewed on a JEM-2000 EX electron microscope at a magnification of  $\times 3,000$ . Ten photographs were randomly chosen from sections of the right auricular cardiocytes of each mouse in accordance with the criteria proposed by Cantin *et al.* [2]. The counting of ANP-granules was done on 25 cm  $\times$  30 cm prints at a final magnification of  $\times 14,000$ , corresponding to an area of 382.7  $\mu\text{m}^2$ . The mean ( $\pm$  SD) of counts was calculated from 50 photographs from five animals of each strain.

**Radioimmunoassay (RIA):** Blood was drawn from a common carotid artery into a syringe containing 1 mg of EDTA and 1,000 units of the kallikrein-inhibitor aprotinin (Bayer, Leverkusen, Germany). The plasma was rapidly frozen and stored at  $-80^\circ\text{C}$  until measurement of the plasma ANP level. The right auricular tissue samples were boiled for 5 min in 10 volumes of 0.1 M acetic acid to abolish intrinsic proteolytic activity. These tissues were then homogenized with a Polytron homogenizer (Kinematica AG, Littau, Lucerne, Switzerland) at 25,000 rpm for 60 sec. Each homogenate was centrifuged at 30,000 g for 30 min at  $4^\circ\text{C}$ , and the supernatants were stored at  $-80^\circ\text{C}$  until RIA. The plasma and auricular ANP levels were measured using an Atrial Natriuretic Factor (rat) RIA kit (Phoenix Pharmaceuticals Inc., Mountain View, CA, U.S.A.).

**Blood pressure (BP) measurement:** The BP was measured in all mice. The systolic BP was measured by the tail-cuff procedure (BP-monitor MK-2000; Muromachi-Kikai Co., Tokyo, Japan). The mice were placed in an equipped holder for 5–10 min until they became calm prior to monitoring their blood pressure with the tail-cuff method. The BP data of mice that could not keep quiet during the measurement was excluded. The data with noise during the pulse wave monitoring of blood pressure was also excluded. The BP values obtained from three consecutive measurements of the respective mice were averaged and recorded at each time point.

**Statistical analysis:** Data were expressed as means  $\pm$  SD. Statistical analysis of the data shown in Table 1 was performed by one-way ANOVA followed by Scheffe type multiple comparison test.  $P$ -Values less than 0.05 were con-

sidered as statistically significant.

Immunoreactivity for ANP (IR-ANP) was found in the right auricular cardiocytes of all mice, and was located primarily in the perinuclear region of the cardiocytes in all strains (Fig. 1). The IR-ANP of the auricular cardiocytes was most intense in ICR (Fig. 1A) strain, and moderate in C57BL (Fig. 1B) and C3H (Fig. 1C) strains. The immunoreactivities in BALB/c (Fig. 1D) and DBA/2 (Fig. 1E) strains were weaker than those in other strains. Ultrastructurally, the right auricular cardiocytes contained a centrally located nucleus, numerous mitochondria, myofibrils, small amount of rough endoplasmic reticulum, Golgi apparatus and electron-dense granules in all strains of mice (Fig. 2). The granules were variable in size and number, and were mainly located in the perinuclear region in association with the Golgi apparatus. The density of granules was greater in ICR (Fig. 2A), and was fewer in BALB/c (Fig. 2D) and DBA/2 (Fig. 2E) than those in C57BL (Fig. 2B) and C3H (Fig. 2C) mice. By ultrastructural morphometry, the number of ANP-granules in the auricular cardiocyte was significantly greater in ICR, and was fewer in DBA/2 than other mice. The number of ANP-granules in C3H and BALB/c mice was greater than those in DBA/2, but was fewer than those in C57BL and ICR mice. In RIA, the auricular ANP content in ICR mice was significantly higher than that in other strains, but the plasma ANP concentrations and blood pressure were comparable in all strains.

Although the ultrastructural evaluation of auricular cardiocytes revealed the existence of electron-dense granules in all strains of mice examined in this study, the number of cellular granules and the atrial IR-ANP content has been found to differ among strains. The numerical differences in ANP-granules paralleled the differences of the immunoreactivity in the cardiocytes among these strains. The number of granules in the auricular cardiocyte has been suggested to be inversely related to the body size in mammals [2, 5]. In our previous reports, however, the number of granules in atrial cardiocytes was not inversely related to the body size in various mammals [9–11]. In the present study, the number of the ANP-granules in C57BL mice was different from that in DBA/2 mice, in spite of their similar body size (average body weight: C57BL, 26.8 g; DBA/2, 27.4 g), thus suggesting that the numerical difference in ANP-granules is unrelated to the body size, at least in mice. We initially speculated that the difference in the number of ANP-granules among the strains of mice was related to their physiological functions.

Numerical changes in the ANP-granules are observed

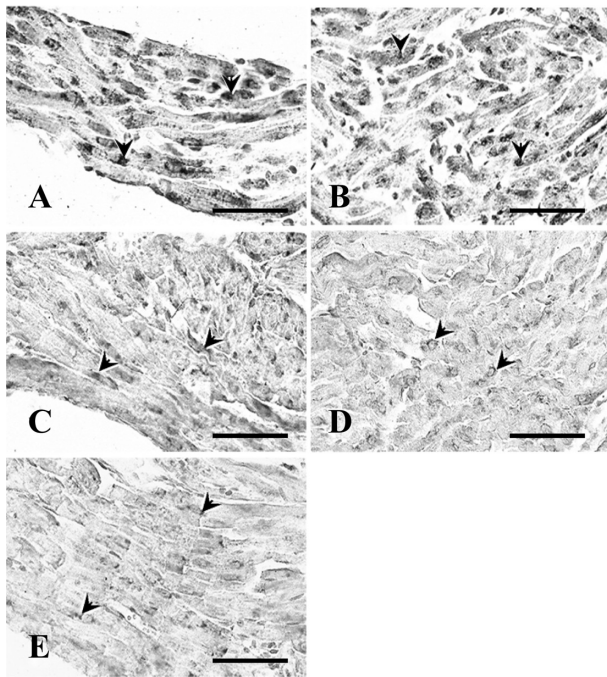


Fig. 1. Immunohistochemical staining of ANP in the right auricular tissue. Immunoreactivity for ANP (IR-ANP) is located primarily in the perinuclear region of the cardiocytes in all strains of mice (arrowheads). IR-ANP is most intense in ICR mice (A), moderate in C57BL/6J (B) and C3H/HeN (C) mice, weaker in BALB/cA (D) and DBA/2Cr (E). Each bar represents 50  $\mu$ m.

under various physiological conditions. For example, the number of ANP-granules in the cell decreases during hypertension and in animals with cardiac abnormalities, while conversely, the plasma ANP concentration and the cellular ANP mRNA levels increase in the presence of such circulatory dysfunction, thus suggesting that numerical changes in the ANP-granules are closely associated with their synthesis and secretion in the cardiocytes, and that their synthesis and secretion are enhanced in the cells with fewer granules [7, 12, 15]. It is therefore possible that the synthetic and secretory ability is enhanced in the strains of mice with fewer granules in the cardiocytes. However, the numerical difference in the ANP-granules in these strains does not seem to provide a quantitative alteration of the intracellular ANP synthesis and release to occur for blood pressure regulation, because the plasma ANP concentrations were similar in all strains, just as the blood pressure was comparable in all mice (Table 1).

The ANP present in the auricle is generally believed to be a hormone with vasodilating and natriuretic activities, and it is stored in the secretory granules and secreted by a "regulated pathway". In this pathway, newly synthesized proteins destined for secretion are stored at high concentrations in the secretory granules until the cell receives an appropriate stimulus [6], such as distension of the atrial wall [4]. If the proteins are secreted as fast as they are synthesized, then the secretory products are hard to find in electron micrographs of the cytoplasm. Therefore, the secretory products are diffusely distributed in the cytoplasm

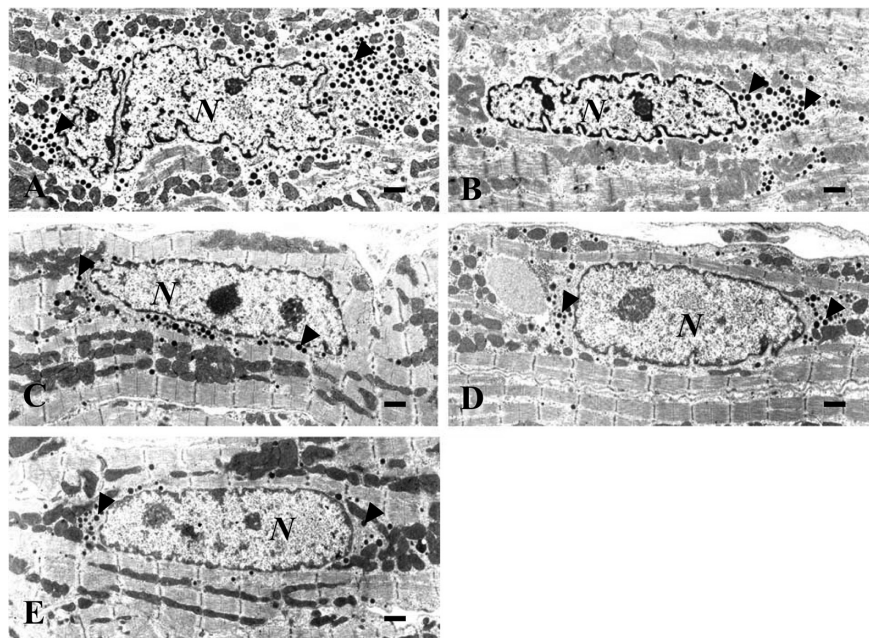


Fig. 2. Electron micrographs of the right auricular cardiocytes in ICR (A), C57BL/6J (B), C3H/HeN (C), BALB/cA (D), and DBA/2Cr (E) mice. In the auricular cardiocytes, ANP-granules are variable in size and number, and are mainly located in the perinuclear region in all strains of mice. The number of granules in ICR was greater, and fewer in BALB/c and DBA/2 than those in C57BL and C3H mice. Each bar represents 1  $\mu$ m. Nucleus (N), ANP-granules (arrowheads).



rather than existing as a granule, thus suggesting that the heart ANP in such cases is rapidly released by a “constitutive pathway” [6]. In the present study, the auricular ANP content did not significantly differ in four strains (C57BL, BALB/c, C3H, DBA/2), although, it did differ in the ICR mice, and the plasma ANP concentration showed a similar value for all mice in the RIA. Regarding the density of the ANP-granules and IR-ANP in the auricular cardiocytes, differences were recognized between the various strains. Consequently, we herein propose that the distribution pattern of ANP-granules in the cardiocytes differs between these strains of mice, and that the ANP in the auricular cardiocytes may be widely distributed in the cytoplasm via a constitutive pathway rather than stored as secretory granules in the auricles of the strain that had fewer ANP-granules with a lower IR-ANP. It was unclear whether the differences in the cellular distribution pattern of ANP-granules among these strains of mice were due to the differences in ANP synthesis and release, or whether they were related to a different physiological mechanism to coordinate the functionality of ANP during the regulation of the blood pressure and volume homeostasis in these strains.

In conclusion, our present findings indicated that the density of ANP-granules and the immunoreactivity for ANP in the auricular tissue are different between the five strains of examined mice. This suggests that there are strain-specific differences in the ANP circulating system in the mice used in this study. Furthermore, such morphological differences in ANP, in spite of the normal blood pressure level in all five of these strains of mice, may be ascribable to differences in the mechanism of cardiac ANP synthesis and secretion in response to blood pressure variations between these mice. We believe our data provide the basis for future biochemical and biophysical studies, and should be taken into account when an ANP study is carried out using one of the strains evaluated in this study. Future studies will be needed to clarify whether such differences between the strains of mice are based on differences in their genetic backgrounds.

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