The Effect of Aging on ANP Levels in the Plasma, Heart, and Brain of Rats

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**Atrial natriuretic peptide (ANP)** is a hormone important in the cardiovascular system via its regulatory roles in sodium and water excretion, and in vasodilatation. Aging represents a major risk factor in the development of hypertension, a perturbation which may activate compensatory mechanisms. The influence of aging on the ANP levels in plasma, atria, ventricles, hypothalamus, and brainstem was evaluated by comparing young (3 mo), middle-aged (12 mo), and old (24 mo) rats. Plasma and ventricular ANP levels increased with age, while ANP content in the atria as well as hypothalamus decreased significantly. PreproANP mRNA contents increased with age in the ventricle but not in the atrium. It is suggested that the increase in plasma ANP levels in old rats is due to the increase in ANP secretion from the atrium and the ventricle, partly as a result of an increase of release of ANP from hypothalamus.

**METHODS**

**Animals.** — Male Sprague-Dawley rats aged 3, 12, and 24 months (young, adult, and old) were housed at a constant temperature (22 °C) on a 12-h dark-light cycle. Food and water were freely available.

**Radioimmunoassay (RIA).** — To assay tissue immunoreactive ANP concentration, the atrium, ventricle, hypothalamus, and brainstem were dissected from decapitated rats, quickly frozen on dry ice, and stored at -70 °C. Tissues were homogenized in 2 N acetic acid and boiled for 10 minutes. Aliquots of 50 μl of homogenate were aspirated for protein assay. The remaining homogenate was centrifuged for 20 minutes at 17,000 × g at 4 °C. The supernatant was lyophilized and then stored at -20 °C until ANP assay. The lyophilized samples were dissolved in RIA buffer. One hundred μl of sample or hANP standard appropriately diluted in assay buffer were incubated with 100 μl of a 1:1000 dilution of rabbit-anti-hANP antiserum (a gift from Dr. T. Yandle, Christchurch, New Zealand) and 100 μl of 125I-hANP (about 10,000 cpm) at 4 °C overnight. Two hundred μl of dextran-coated charcoal suspension (1.5%) was added to separate bound and free ligand. 125I-hANP was iodinated in our laboratory (Tang et al., 1988), and the antiserum cross-reacted about 70% with rat ANP but not with brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP).

**Protein measurements.** — Fifty μl of homogenate or standard (bovine serum albumin) were boiled with 1 N NaOH for 10 minutes, and 50 μl of this boiled sample was mixed with 2.5 ml of protein assay reagent (Bio-Rad, Hercules, CA). After 10 minutes of incubation at room temperature, samples were measured spectrophotometrically at 595 nm.

**Plasma immunoreactive ANP measurements.** — Trunk blood was collected into plastic tubes containing EDTA and centrifuged at 2,000 × g for 20 minutes. Plasma was aspirated and stored at -70 °C until use. For the extraction of ANP, 2 ml plasma was acidified with 1% trifluoroacetic acid (TFA) and passed through a Sep-column pretreated with 1 ml acetonitrile-1%TFA (3:2 by volume) and 9 ml 1% TFA. The peptide was slowly eluted with 3 ml acetoni-
trile-1%TFA (3:2 by volume). The eluates were evaporated to dryness in a speed-vac concentrator. Samples were reconstituted in 0.6 ml assay buffer, and 50 μl was used for ANP assay.

Solution hybridization–RNase protection assay for heart ANP mRNA. Atrial and ventricular total RNA was extracted by Trizol-reagent (Life Technologies, Gaithersburg, MD). Plasmid preproANP DNA and β-actin DNA (both kindly provided by Dr. D. J. Autelitano, Baker Medical Research Institute, Prahran, Australia) were transformed into E. coli JM109. After harvest, these plasmid DNAs were linearized with restriction enzymes (PreproANP: Eco RI for synthesis of probe and Sal I for standard; β-actin: Eco RI for probe and Hind III for standard). The standard RNA and the riboprobes were synthesized using polymerases (preproANP: T7 polymerase for probe and SP6 polymerase for standard; β-actin: SP6 polymerase for probe and T7 polymerase for standard) and reagents available in a kit obtained from Promega (Madison, WI). The sizes of the riboprobes for preproANP and β-actin were 755 and 387 nucleotides, respectively. Ventricular RNA (10 μg) were co-hybridized with preproANP and β-actin riboprobes. For the atrium, 0.1 μg of total RNA was used for preproANP mRNA assay while 10 μg were used for β-actin mRNA assay. The details of hybridization and separation of the hybrids by polyacrylamide gel electrophoresis have been described (Tang and Lau, 1996). The hybrids were cut out for scintillation counting using the X-ray film as templates. The values of preproANP and β-actin mRNA in the sample were read off from the standard curves, and atrial and ventricular preproANP mRNA contents were expressed as pg preproANP mRNA per pg β-actin mRNA.

Statistical analysis. The results were analyzed using one-way analysis of variance (ANOVA), and multiple comparison procedure was performed by Tukey’s test, taking p < .05 as the level of significance. Data for plasma, atrial, and hypothalamic ANP levels were also subjected to correlation analysis, and p < .05 was considered significant. In addition, the correlations of ANP levels between atrium and plasma, and between atrium and hypothalamus, were calculated for each age group and then pooled. Analysis of covariance and multiple regression analysis were also performed on these data.

RESULTS

The ANP levels in the plasma, in the heart, and in brain tissues are shown in Table 1. By ANOVA, it was found that there were significant age-related differences in ANP levels in the plasma, atrium, and the hypothalamus. ANP levels in the plasma of old rats were significantly higher than those in the young and middle-aged rats (p < .05). On the other hand, atrial ANP level in young rats (3 mo) was higher than in old rats (24 mo) (p < .05). In contrast, both ventricular ANP concentration and total ventricular ANP content increase with age. The ratio of total ventricular ANP over atrial ANP is very low. It is interesting that ANP contents in hypothalamic of middle-aged and old rats were also significantly decreased (p < .01). Correlation test showed that atrial ANP was correlated negatively with plasma ANP (r = −.627, p < .001) (Figure 1A) and positively with ANP content in the hypothalamus (r = .450, p < .02) (Figure 1B). Further analysis, however, indicated that after removing the effect of age, only the association between plasma ANP and atrial ANP was significant (r = −.371, p = .072, pooled correlation from each age group; p = .048, analysis of covariance; p = .034, multiple regression).

The X-ray film of polyacrylamide gel electrophoresis of RNA hybrids of ventricular preproANP is shown in Figure 2 while the levels of preproANP mRNA in both the ventricle and the atrium are shown in Table 2. There was a significant age-related increase of preproANP mRNA contents in the ventricle but not in the atrium.

DISCUSSION

Many studies have documented an increased plasma level of ANP in healthy elderly humans compared with the level of the average population (Haller et al., 1987; Morrow et al., 1989; Clark et al., 1990). Possible explanations for this finding include chronic volume expansion due to occult congestive heart failure or impaired renal sodium excretion in the elderly (Ezaki et al., 1988; Mukerrin et al., 1993). Both possibilities involved the release of ANP from the atria. It is well known that, in the aged, there is a fundamental alteration in renal sodium homeostasis due to a dif-

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Table 1. The Effect of Aging on ANP Levels in the Plasma (pg/ml), Atria (μg/mg protein), Ventricles (pg/mg protein), Hypothalamus, and the Brainstem (pg/mg protein)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>3 Months (Young)</th>
<th>12 Months (Mature)</th>
<th>24 Months (Old)</th>
<th>ANOVA p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (n = 10)</td>
<td>26.7 ± 5.2</td>
<td>36.8 ± 3.7</td>
<td>55.0 ± 5.66</td>
<td>.003</td>
</tr>
<tr>
<td>Atria (n = 8)</td>
<td>0.77 ± 0.05</td>
<td>0.63 ± 0.03</td>
<td>0.50 ± 0.05</td>
<td>.018</td>
</tr>
<tr>
<td>Ventricles (n = 7-8)</td>
<td>66.29 ± 4.47 (8)</td>
<td>81.99 ± 4.97 (7)</td>
<td>128.4 ± 13.6 (8)*</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hypothalamus (n = 10)</td>
<td>318.0 ± 12.5</td>
<td>226.4 ± 8.0*</td>
<td>214.9 ± 13.8*</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Brainstem (n = 10)</td>
<td>21.3 ± 1.8</td>
<td>17.6 ± 1.31</td>
<td>18.1 ± 1.4</td>
<td>.596 (n.s.)</td>
</tr>
<tr>
<td>Total atrial ANP (μg) (n = 8)</td>
<td>5.82 ± 0.32</td>
<td>7.92 ± 0.75</td>
<td>6.82 ± 0.62</td>
<td>.056 (n.s.)</td>
</tr>
<tr>
<td>Total ventricular ANP (ng) (n = 7-8)</td>
<td>5.45 ± 0.14 (8)</td>
<td>12.31 ± 0.21* (7)</td>
<td>20.04 ± 2.56* (8)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

* p < .05 compared with 3 months old.
* p < .05 compared with 12 months old.
minimized glomerular filtration rate and a less active renin-aldosterone system (Luft et al., 1979). It has also been demonstrated that aged individuals have defects in sodium excretion after acute sodium loading (Luft et al., 1979, 1982). The kidney is the main site for ANP catabolism (Tang et al., 1984; Berg et al., 1988; Sonnenberg et al., 1988), and reduced renal function in aging may contribute to the high plasma ANP concentration. Reduced ANP clearance does not necessarily account for the increased ANP level, because the plasma ANP concentration in patients with advanced chronic renal failure was not significantly higher than that in normal subjects (Rascher et al., 1985).

We observed an age-associated increase in plasma ANP level in the rats, in agreement with the studies of Korytkowski and Landenson (1991) and Kao et al. (1992). Whereas Thibault et al. (1989) suggested a considerable contribution of heart ventricles to plasma ANP level in cardiomyopathic hamsters with heart failure, we are of the opinion that both atrium and the ventricle may contribute significantly to the increase of plasma ANP levels in aging rats. Similar to the findings of Younes et al. (1995), we found an increase in both ANP contents and preproANP mRNA levels in the ventricle with aging; but in contrast to the observation of Hong et al. (1992), there was no decrease in preproANP mRNA levels in the atrium (although atrial ANP content decreased). It is generally accepted that changes in mRNA content are a good indication of changes in peptide synthesis. A decrease in atrial ANP content together with a lack of change in preproANP mRNA level would indicate an increase in ANP release. Similarly, an in-

Figure 1. (A) The correlation of atrial ANP and plasma ANP: plasma ANP is negatively correlated with atrial ANP. \( r = -0.627, \ p < .001 \) (pooled from each age group, \( r = -0.371, \ p = .072 \)). B. The correlation of atrial ANP and hypothalamic ANP: atrial ANP is positively correlated with hypothalamic ANP. \( r = 0.45, \ p < .02 \) (pooled from each age group, \( r = -0.301, \ p = .122 \)).

Figure 2. The X-ray film of polyacrylamide gel electrophoresis of RNA hybrids of preproANP and \( \beta \)-actin mRNAs with \( ^{32} \)P-labeled preproANP and \( \beta \)-actin probes after overnight exposure. The upper bands are the preproANP mRNA hybrids, and the lower bands are the \( \beta \)-actin mRNA hybrids. Lane P is the riboprobes; lanes 1 to 5 are the standards (for preproANP mRNA: 0, 10, 50, 100, 500 pg; for \( \beta \)-actin mRNA: 0, 5, 10, 50, 100 pg). Y lanes with RNA samples from 3-month-old rats, M lanes from 12-month-old rats, and O lanes from 24-month-old rats.

Table 2. PreproANP mRNA Levels (pg/pg actin) in the Atrium and the Ventricle of Aging Rats

<table>
<thead>
<tr>
<th></th>
<th>3 Months (Young)</th>
<th>12 Months (Mature)</th>
<th>24 Months (Old)</th>
<th>ANOVA ( p )-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrium</td>
<td>1392 ± 360.7 (5)</td>
<td>1361 ± 284.2 (5)</td>
<td>1531 ± 506.4 (4)</td>
<td>.948 (n.s.)</td>
</tr>
<tr>
<td>Ventricle</td>
<td>1.758 ± 0.225 (8)</td>
<td>3.366 ± 0.517 (7)</td>
<td>3.641 ± 0.518 (8)</td>
<td>.012</td>
</tr>
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</table>

Note: All values are mean ± SE; the number of samples per age group are in parentheses. *\( p < .05 \) compared with 3 months old.
crease in ventricular ANP mRNA content with an increase in peptide content would indicate the same. The magnitudes of increase for the peptide and mRNA contents in the ventricle were about the same, i.e., twofold, meaning that the increase in release might be twofold. Therefore, we conclude that both the atrium and the ventricle contribute significantly to the age-related increase in plasma ANP levels and that correlation study between peptide and release is only valid when there is no change in peptide synthesis.

The possibility of increased atrial ANP release with aging is strongly indicated, although with aging a decreased spontaneous release of ANP and a decreased release of ANP in response to acute left atrial stretch (Opie et al., 1992) have been reported. This is based on additional evidence presented below. First, the study by Opie et al. was done in vitro and it may be different from the situation in vivo. Second, although direct atrial stretch is an important stimulus for ANP secretion, other factors such as the central nervous system and hormones or other humoral agents may contribute to the regulation of ANP secretion. Evidence for a stimulatory role of the CNS in plasma ANP regulation came from blocking the effect of central ANP, either by central injection of ANP antiserum (Charles et al., 1991) or by lesions of specific hypothalamic areas (AV3V) (Rauch et al., 1990), which could inhibit the plasma ANP response to plasma volume expansion and/or to hypertonic saline infusion. Evidence for the latter comes from the study of Tummalala et al. (1992), who reported that atria from aged rats has an increased response to phenylephrine compared with young rats, although the secretary response to stretch was less than that of young rats.

In addition to the report of an age-related decrease in atrial ANP, we also observed a significant decrease of hypothalamic ANP with aging. The decrease in ANP levels in the hypothalamus could be due to an increase in ANP release or a decrease in synthesis, or both. However, the effect of aging on brain proANP mRNA is not known. The involvement of hypothalamic ANP in the regulation of atrial ANP has been reported by Antunes-Rodrigues et al. (1991, 1993). Charles et al. (1991) also postulated that hypothalamic ANP may inhibit the vagal tone via projection to the brainstem to increase atrial ANP secretion, in view of the evidence that vagal tone suppresses cardiac ANP secretion (Phillips et al., 1989). If the decrease of hypothalamic ANP is the result of increased secretion, it is possible that the increased ANP release from the atrium may not be the result of a change in response to atrial stretch but more likely due to the change of central ANP modulation in aging rats. This speculation is supported by the correlation test, which showed that atrial ANP was positively correlated with hypothalamic ANP \( (r = 45, p < 0.05) \) although the pooled correlation from each age group was not significant \( (r = 0.301, p = 122) \). More experiments are needed to confirm the role of hypothalamic ANP on plasma ANP levels during aging.

**ACKNOWLEDGMENTS**

We are grateful to Dr. Tim Yandle (Christchurch, New Zealand) for the gift of hANP anti-serum and to Dr. Dominic Autelitano (Prahran, Australia) for the gift of preproANP cDNA and B-actin cDNA used in this study. We would also like to thank Dr. C. J. Lloyd, the Department of Statistics, The University of Hong Kong, for his help in the correlation analysis within each age group, the analysis of covariance, and the multiple regression analysis.

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