Alterations in Atrial Natriuretic Peptide (ANP) Secretion and Renal Effects in Aging

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Atrial natriuretic peptide (ANP) is a hormone secreted by the heart that increases salt and water excretion by the kidneys. The primary physiological stimulus for the release of this peptide appears to be atrial stretch (Dietz, 1984; Dietz et al., 1991), and its secretion is modulated by several other hormones including endothelial-derived factors (Ruskoaho, 1992). For example, endothelin is a potent secretogogue (Skvorak et al., 1995), and nitric oxide is a potent inhibitor (Melo and Sonnenberg, 1996). ANP has been shown to play an important role both in blood volume homeostasis (Hirth et al., 1986) and in the normal regulation of arterial blood pressure (Pannanai et al., 1988). Chronic changes in ANP secretion have been demonstrated in pathophysiological conditions such as heart failure or kidney diseases (Suda et al., 1988; Cody, 1990). In animal experiments, overexpression of the ANP gene results in natriuresis and hypotension, whereas a reduction in gene expression leads to sodium retention and hypertension (Field et al., 1991; John et al., 1995).

One of the earliest and most consistent findings concerning ANP and aging was that plasma levels are elevated with age in both humans (Tonolo et al., 1989) and rats (Korytkowski and Ladenson, 1991). Basal ANP secretion rate from isolated atria has been found to be both increased and depressed (Morrow et al., 1991; Tummala et al., 1992), whereas the ANP response to adrenergic stimuli has been reported to be increased (Tummala et al., 1992). Both ANP and proANP concentrations in the heart appear to be increased with age (Korytkowski and Ladenson, 1991; Giordano et al., 1993), whereas ANP mRNA has been reported to be decreased with age (Hong et al., 1992). ANP secretion in response to atrial stretch appears to be unaltered in aging (Tummala et al., 1992), but proANP secretion has not been studied in aging.

The biological effects of ANP also appear to be altered in aging. The hypotensive action of ANP appears to be attenuated (Mulkerrin et al., 1993), which could contribute to increased blood pressure with aging. At least two studies have suggested increased diuretic and natriuretic responses to ANP in aged rats (Depriest et al., 1990) and in humans (Tajima et al., 1988). An increased sensitivity to ANP could contribute to hyponatraemia, which is common in elderly persons.

The purposes of this study were, first, to compare the effects of volume expansion on sodium excretion in young and aged rats and to evaluate the role of the atria in this response. Second, we compared the effects of a standardized ANP injection on sodium excretion in young and aged rats, both intact and atrial appendectomy. Third, we compared the effects of two stimuli of ANP secretion in young and aged rats. We chose atrial stretch and endothelin because the former is a mechanical stimulus (Dietz, 1984), while the latter is a receptor-mediated stimulus (Skvorak et al., 1995).
Materials and Methods

Protocol for in vivo Blood Volume Expansion Experiments

Young (2–3 mo) and old (18–20 mo) male Sprague-Dawley rats were anesthetized with sodium pentobarbital (50 mg/kg) and were given supplemental anesthetic as needed. The rats were placed on a heating pad to maintain body temperature between 37–38 °C. Following tracheal cannulation, polyethylene catheters were placed in the right jugular vein (PE-50, for infusions), in the right carotid artery (PE-50, for blood sampling and blood pressure monitoring), and in both ureters (PE-10, for urine collection). Rats were placed on a constant volume ventilator prior to sternotomy and pericardiectomy.

For the rats receiving bilateral atrial appendectomies (young, Group 2, n = 9, and old, Group 4, n = 7), both atrial appendages were clamped gently with hemostatic forceps, ligated, and excised. In the rats with sham atrial appendectomies (young, Group 1, n = 8, and old, Group 3, n = 8), the atrial appendages were gently pinched with hemostatic forceps for 20 sec and then released. The chest of each rat then was covered with gauze soaked in 0.9% saline.

A one-hour equilibrium period followed the surgical preparation, and data were gathered over six 20-min urine collection periods. At 40 min, all rats received a 20-min iv infusion of 6% albumin in Krebs solution sufficient to expand estimated blood volume by 15%. Blood volume was estimated as 6% of body weight. A study by Kaler and Neaves (1981) demonstrated that in male Sprague-Dawley rats between 3–24 mo of age (ranging in body weight from 419–702 g), blood plasma volume increased in proportion to body weight. Mean arterial blood pressure was measured throughout the experiment with a pressure transducer, recorded and displayed using Windaq Analysis Software (DATAQ Instruments, Akron, OH) and averaged for each 20-min period. Urine was collected in preweighed tubes at 20, 40, 60, 80, 100, and 120 min. Arterial blood samples of 2 ml each were drawn from the carotid artery catheter 5 min before the infusion, as well as 5 min after and 30 min after the infusion. Each blood sample was replaced simultaneously with an equal volume of 6% albumin in Krebs solution (iv) to avoid acute changes in blood volume during blood sampling. At 100 min, each rat was injected with ANP (1 µg/kg) to determine its renal response to a standardized dose. All blood samples were placed immediately in prechilled tubes containing EDTA. Plasma obtained after centrifugation was frozen at -20 °C until extraction and assay.

Protocol for Isolated Atria

Details of the atrial perfusion technique are provided elsewhere (Dietz et al., 1991; Skvorak et al., 1995). Young (2–3 mo, n = 9) and old (18–20 mo, n = 8) male Sprague-Dawley rats were weighed and anesthetized with sodium pentobarbital (50 mg/kg). The chest was opened and the left atrium was removed and placed in cold, oxygenated Krebs buffer. The atrium was carefully dissected and secured to a perfusion cannula with 4-O silk. The atria were placed in a reservoir tube containing 4 ml of modified Krebs buffer perfusate warmed to 38 °C and gassed with 95% O₂–5% CO₂. A pump moved fluid from the reservoir through the atria and back to the reservoir continually at a rate of 0.25 ml/min. All atria were paced at 200 beats/min.

Atrial pressure, used as an index of atrial stretch, was measured with a pressure transducer and displayed using the data acquisition system. The pressure in the atria could be adjusted by changing the height of the atrial outflow cannula. Atrial pressure was maintained at a 2–3 mmHg baseline level for three 10-min equilibration periods, during which time the fluid in the reservoir was changed but discarded. The experiment consisted of three 10-min periods where atrial pressure was maintained at a 2–3 mmHg baseline level and then the atrial pressure was increased to 8–10 mmHg for six additional 10-min periods (distension). During each of the last three 10-min periods, endothelin was added to the perfusate at a concentration of 50 nM. In previous studies we found that this concentration of endothelin produced a marked stimulation of ANP secretion in stretched atria (Skvorak et al., 1995). Reservoir perfusate removed at the end of each 10-min period was saved and frozen at -20 °C for later analysis.

Assays and Statistics

Atria were extracted as reported previously (Dietz et al., 1995). ANP was measured in perfusates, plasma, or atrial extracts by radioimmunoassay using the methods previously described in our laboratory (Dietz et al., 1991, 1995). For the volume expansion experiments, samples of rat plasma (0.5 ml) were extracted using Amprep C 8 cartridges (Amersham, Arlington Heights, IL) and assayed in duplicate. For the atrial perfusion experiments, two 50 µl samples were assayed directly by radioimmunoassay without prior extraction as we have done in several previous studies. Urine sodium and potassium concentrations were measured by flame photometry (model 943, Instrumentation Laboratories, Lexington, MA).

Statistical significance was assessed using a two-way analysis of variance (ANOVA) with repeated measures on one factor (group as one factor and time as the other) for data in Figures 1, 2, 4, and 6 and Table 1. A one-way ANOVA was used to analyze the data in Figures 3 and 5. All post-hoc tests were assessed using the Student-Newman-Keuls test. In all cases p < .05 was considered the criterion for statistical significance.

Results

The results for sodium excretion with volume expansion and ANP injection are shown in Figure 1. These data were not factored by body weight. The young rats are shown in the upper panel and the old rats in the lower panel. Volume expansion resulted in a significant increase in sodium excretion rate in all four groups. In the young control rats, sodium excretion rate increased approximately fivefold with volume expansion and an additional twofold with the ANP injection. In the young atrial appendectomized rats, volume expansion and the ANP injection each resulted in significant increases in sodium excretion rate. However, the increases in sodium excretion rate were significantly greater in the intact rats compared to the appendectomized (p < .05). In the old intact rats, volume expansion and ANP injection resulted in significant increases in sodium excre-
Figure 1. Effect of acute volume expansion on sodium excretion rate. Top panel shows the response to volume expansion was significantly attenuated in young atrial appendectomized rats (Group 3, n = 9) compared to young control rats (Group 1, n = 8). Values are means ± SEM, *p < .05, a significant difference between the intact and appendectomized rats. Volume expansion resulted in a significant increase in sodium excretion rate in all four groups compared to their own control values (all p < .05). Bottom panel shows no significant difference in the sodium excretion response to volume expansion comparing old appendectomized (Group 4, n = 7) with control rats (Group 3, n = 8).

Figure 2. Effect of acute volume expansion on sodium excretion rate factored by kilogram body weight. Top panel shows that sodium excretion in response to volume expansion was significantly attenuated in young atrial appendectomized rats (Group 2, n = 9) compared to young control rats (Group 1, n = 8). Values are means ± SEM, *p < .05, a significant difference between the intact and appendectomized rats. Bottom panel shows no significant difference in the sodium excretion response to volume expansion between old atrial appendectomized (Group 4, n = 7) and control rats (Group 3, n = 8).

The response to volume expansion and ANP injection in old appendectomized rats appeared to be much lower than the response in intact old rats, but the difference was not statistically significant (p = .06). Because of the size difference between the older and younger rats (407 ± 12 g vs 744 ± 31 g, p < .05), the older rats received more volume during the volume expansion and more ANP for the injection. Therefore, we also analyzed the sodium excretion data factored by body weight (Figure 2). When factored by body weight, the young intact rats showed a significantly greater response to volume expansion when compared to the young appendectomized rats and both intact and appendectomized older rats (p < .05, ANOVA). These results show that the renal response to volume expansion is blunted with either appendectomy or aging. In fact, in aged rats with the appendectomy, this response was nearly abolished (Figure 2, lower panel). Baseline sodium excretion (0–40 min) appeared to be greater in the young rats com-
Table 1. The Effects of Volume Expansion and ANP Injection on Mean Arterial Blood Pressure in Young and Old Rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>0-20 min</th>
<th>20-40 min</th>
<th>40-60 min</th>
<th>60-80 min</th>
<th>80-100 min</th>
<th>100-120 min</th>
</tr>
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<tbody>
<tr>
<td>Young Control</td>
<td>122 ± 6</td>
<td>120 ± 6</td>
<td>129 ± 5</td>
<td>130 ± 5</td>
<td>119 ± 6</td>
<td>114 ± 5</td>
</tr>
<tr>
<td>Young Appendectomy</td>
<td>124 ± 4</td>
<td>123 ± 4</td>
<td>126 ± 4</td>
<td>128 ± 4</td>
<td>115 ± 5</td>
<td>108 ± 6*</td>
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<tr>
<td>Old Control</td>
<td>122 ± 2</td>
<td>122 ± 2</td>
<td>131 ± 5</td>
<td>132 ± 3</td>
<td>125 ± 3</td>
<td>118 ± 3</td>
</tr>
<tr>
<td>Old Appendectomy</td>
<td>126 ± 4</td>
<td>124 ± 2</td>
<td>132 ± 6</td>
<td>135 ± 5</td>
<td>126 ± 5</td>
<td>117 ± 5</td>
</tr>
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Notes. ANP = atrial natriuretic peptide; VE = period of blood volume expansion; Values are means ± SEM.
*p < .05 statistically significant difference for a value from its own control period (0-20 min). There were no significant differences between the groups.

Figure 3 shows the change in sodium excretion with the ANP injection both as raw values (upper panel) and factored by body weight (lower panel). These data represent the difference between urine collection periods 5 (80–100 min) and 6 (100–120 min). Atrial appendectomy reduced the response to the ANP injections in both young (p < .05) and old (p < .05) rats. There was no significant difference in the change in sodium excretion with ANP between the intact young and old rats. However, when the sodium excretion data are factored by body weight (lower panel), the young intact rats showed a larger response to exogenous ANP than any of the other groups (p < .05).

The plasma concentrations of ANP during the volume expansion are shown in Figure 4. Plasma ANP concentration tended to increase with volume expansion in all groups compared to their own controls (35 min sample). ANP was significantly increased in the young control rats at both 65 and 95 min and in the old control rats at 65 min (all p < .05). Plasma ANP concentration tended to be lower in the appendectomized rats, but the differences were not statistically significant. Also, there were no significant differences in plasma ANP concentration between the young and old rats (p > .05).

Figure 5 compares proANP stored in atria of young and old rats. These values were derived from the ANP assay since our previous data have shown that the major storage form of ANP is as proANP 1-126 (Dietz et al., 1995). We recently have found that the antibody used for the ANP assay (Peninsula Laboratories, Belmont, CA: RAS 8798) shows a 70.2% cross-reactivity with the 1-126 a.a. prohormone. These data show that the proANP concentration is significantly lower (p < .05) in the atria of the old rats compared to the atria of young rats. However, the total proANP is actually greater (p < .05) in the older rats compared to the young rats. This was due to the much larger atrial size in the older rats.

Figure 6 shows data from the isolated perfused atria experiments. The upper panel shows intra-atrial pressure, which was regulated at 2 mmHg for the first 3 periods and increased to approximately 9 mmHg for the last six periods.
Figure 4. Plasma concentrations of ANP during the volume expansion. Values are means ± SEM. Plasma ANP concentration tended to increase with volume expansion in all groups compared to their own controls (35 min sample). ANP was significantly increased in the young control rats at both 65 and 95 min and in the old control rats at 65 min (all p < .05). Also, there were no significant differences in plasma ANP concentration between the young and old rats (p > .05).

Figure 5. A comparison of proANP 1-126 concentration and content in atria of young (n = 11) and old (n = 13) rats. These data show that the proANP concentration is significantly lower (p < .05) in the atria of the old rats. However, the total proANP is actually greater (p < .05) in the old rats compared to the young rats. Values are means ± SEM.

Figure 6. A comparison of the effects of stretch (pressure) and endothelin on ANP secretion from the isolated perfused atria of young (n = 9) and old (n = 8) rats. The top panel shows intra-atrial pressure which was regulated at 2 mmHg for the first 3 periods, and increased to approximately 9 mmHg for the last 6 periods. There were no significant differences in atrial pressure between the old and young rats. The bottom panel shows ANP secretion as a percentage of baseline. The atria from the young rats show a dramatic increase in ANP secretion in response to atrial stretch and further marked increase in secretion in response to endothelin. Both of these responses were markedly attenuated in the old rats (p < .05). Values are means ± SEM. *p < .05, a significant difference in the ANP secretion rates between the young and old rats.

It is apparent that there were no differences in atrial pressure between the old and young rats. The lower panel shows ANP secretion rate as a percentage of the baseline, i.e., the third low-pressure period. We have used this method of calculating data routinely because of the variability in basal ANP secretion rates (Skvorak et al., 1995, 1996). Basal ANP secretion rate (Figure 6, the third control period) was 120 ± 12 pg/min in atria from rats and 144 ± 20 pg/min in atria from old rats. These values were not significantly different (t = 1.0996 and p = .29). Both groups showed significant increases in ANP secretion compared to their own control values (30 min, p < .05) in response to
atrial stretch and endothelin. The atria from young rats show a dramatic increase in ANP secretion in response to atrial stretch and further marked increase in secretion in response to endothelin. Both of these responses were markedly attenuated in the old rats (p < .05). In the old rats, the ANP response to endothelin + stretch was not significantly greater than the response to stretch alone. Our previous studies show that ANP secretion in the isolated perfused atria is very stable over this time course (Dietz et al., 1991).

**DISCUSSION**

Our results show that both ANP secretion and the renal response to ANP are attenuated in aged rats. In the first experiment, the 15% blood volume expansion resulted in the expected natriuretic response in young intact rats (Figure 2) and a significant increase in plasma ANP levels (Figure 4). However, the natriuretic response to volume expansion was markedly attenuated in the atrial appendectomized rats. ANP levels tended to be less in the appendectomized rats, but the differences did not achieve statistical significance. This may be due to the variability of the hormone measurements or may suggest that reductions in factors other than ANP contribute to the attenuated natriuretic response with atrial appendectomy. Our results in the young rats are very similar to those of Villarreal and colleagues (1986), who compared volume expansion in intact and bilaterally appendectomized young rats. Since there were no differences in blood pressure, we have concluded that the differences in sodium excretion cannot be attributed to hemodynamic changes. Taken together, the results clearly show that the atrial appendages are essential for the normal response to acute volume expansion. In the present study, volume expansion in the aged rats resulted in an attenuated natriuretic response, compared to the young rats (Figure 1). This finding is even more obvious when the responses are factored by body weight (Figure 2), which is critical since the volume expansion stimulus was also factored by body weight.

The natriuretic response to exogenous ANP was attenuated in young appendectomized rats compared to the intact young rats, and was also attenuated in both groups of old rats. Again, these differences are most obvious when the natriuretic responses are compared by body weight (Figure 3, lower panel). Two conclusions can be drawn from these injections. First, the renal response to ANP is greatly reduced in aged rats, and second, some factor or factors from the atria are necessary for the normal natriuretic response to ANP. Such factors may include peptides released from the N-terminus of the ANP prohormone which have been shown to have natriuretic effects in both animals (Dietz et al., 1994) and humans (Vesely et al., 1994). It is possible that the gradual increase in plasma ANP levels seen in aging humans represents a homeostatic adaptation to the reduced sensitivity in the kidney (Tonolo et al., 1989). Our findings disagree substantially with those of Depriest et al. (1990), who found an enhanced natriuretic response to "low dose" ANP in old rats compared to young rats. This could be explained by the fact that their animals were conscious, whereas our animals were anesthetized. The main reason for the response difference that they observed between young and old rats was the lack of a significant response in young rats. However, the dose of ANP that they used (80 ng/Kg/min) is, in fact, not a low dose at all and in our hands (Dietz et al., 1994) and many others produces an increase in sodium excretion of five- to tenfold. Of interest is the finding that the hemodynamic response to ANP is also reduced in older humans (Mulkerin et al., 1993).

Several studies have shown that plasma ANP levels are elevated in aging (Korytkowski and Ladenson, 1991). Some studies indicate that this increase is related to an increase in arterial pressure which leads to ventricular hypertrophy. Therefore, the increased plasma ANP may be the result of increased ventricular secretion of ANP. In our study we found no significant differences in plasma ANP levels between old and young rats (Figure 4). There are substantial differences between our study and those of others (e.g., Korytkowski and Ladenson, 1991), which could account for this discrepancy. Our animals were anesthetized, open chest and ventilated. These procedures would tend to normalize both arterial and intracardiac pressures. For example, mean arterial pressure was not significantly different between our young and old groups (Table 1). Therefore, one could argue that our model does not adequately represent the physiological conditions needed to evaluate the natriuretic system. However, the sham appendectomized young rats were also anesthetized, open chest and ventilated, but they showed marked natriuretic responses to volume expansion and ANP injection. It remains to be determined whether the changes in sensitivity to ANP observed in the old rats are sufficient to exacerbate hypertension and whether the elevated ANP observed in humans is the result of the increased arterial pressure that accompanies aging.

Our previous work using high-performance gel permeation chromatography has shown that the major storage form of ANP is proANP-1-126 (Dietz et al., 1995). Therefore, the atrial concentration data are reported as proANP levels. ANP prohormone concentration was significantly lower in aged rats compared to young rats, but the total prohormone present was actually significantly greater in aged rats (Figure 5). This apparent discrepancy is due mainly to the much greater atrial size in the aged rats.

In the final phase of this study, we compared the secretion of ANP to atrial stretch, a mechanical stimulus (Dietz, 1984), and endothelin, a receptor-mediated stimulus (Skvorak et al., 1995). Although stretch and endothelin produced detectable responses in both young and aged rats, the responses were markedly attenuated in the aged group (Figure 6, lower panel). This difference occurred in spite of the fact that the atrial pressures were identical in both groups (Figure 6, upper panel). The attenuation of ANP secretion in the aged rats is even more surprising when one considers that the total amount of ANP in the aged rats was found to be increased (Figure 5). Opie and colleagues (1992) also found less release of ANP from isolated hearts in response to hypertonic saline. Others have found no change in ANP release in response to volume stimulation or stretch (Morrow et al., 1989; Tummala et al., 1992). However, the present study employed the isolated perfused atria where the experimental design allows a more accurate comparison of these stimuli since atrial pressure can be regulated (Figure 6, upper panel) and environmental factors carefully regulated.
It is not surprising that responses to mechanical stretch and endothelin might both be attenuated in aging since, as we have previously demonstrated (Skvorak et al., 1995, 1996), these stimuli probably share a common final pathway. Both stimuli may require an increase in prostaglandin synthesis and a reduction in nitric oxide production. It is tempting to speculate that these paracrine regulators of ANP secretion may be altered in aging. ANP in the plasma is elevated in aged individuals (Tonolo et al., 1989; Kortykowski and Ladenson, 1991), but this appears to occur despite the fact that ANP secretion is less responsive to stimuli. This finding might suggest that the primary factor responsible for the elevated plasma ANP levels is a decrease in ANP metabolism resulting in a longer half-life (Yamada and Yoshida, 1989).

In summary, we found that both the secretion and renal actions of ANP are attenuated in aged rats. Since normal ANP function has been shown to be critical for the normal regulation of blood volume and blood pressure, these studies suggest that changes in ANP secretion or actions could exacerbate hypertension and heart failure in aged individuals.

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