Ventricular Atrial Natriuretic Factor in the Cardiomyopathic Hamster Model of Congestive Heart Failure


Cardiac atrial, but not ventricular, myocytes contain granules that morphologically resemble secretory granules seen in cells that produce peptide hormones. Involvement of these granules in water and electrolyte homeostasis was suggested by the observation that atrial granularity decreased with sodium loading and increased with sodium restriction and water deprivation. Atrial, but not ventricular, extracts were found to increase urinary sodium and water excretion, and a family of peptides known collectively as atrial natriuretic factors (ANF) were isolated from atrial tissue (for reviews see Flynn and Davies, Ballermann and Brenner, and Needleman et al). Despite the absence of granules in ventricular myocytes, very small amounts of ANF messenger RNA (mRNA) and immunologically detectable ANF have been observed in rat ventricle.

ANF is not only a potent natriuretic and diuretic agent, it also has a number of other properties that suggest that it may work to alleviate extracellular volume expansion. It inhibits smooth muscle contraction, lowers plasma renin levels, and inhibits aldosterone release. ANF is released in response to increased atrial pressure, and the plasma concentration of ANF is elevated in conditions of extracellular volume expansion. In congestive heart failure where fluid retention occurs secondarily to poor renal perfusion, plasma ANF levels increase with the severity of the disease and correlate with increases in cardiac filling pressures. To better understand the role of this endogenous peptide and to evaluate the role of exogenous ANF in the treatment of congestive heart failure, it is important to understand the production, storage, and release of ANF in this disease.

The hereditary, dilated cardiomyopathy of the golden Syrian hamster was chosen as an appropriate animal model of congestive heart failure to study the alterations in ANF storage and production in heart failure. Interest in ANF involvement in the cardiomyopathic hamster began with the observation that bioassayable ANF in the atria decreased with the development of heart failure in these animals. Subsequently shown that plasma ANF levels were elevated and atrial ANF concentration (per milligram of tissue) and ANF specific granularity were decreased. However, due to increased atrial weight of the cardiomyopathic hamster, total atrial content of ANF remained unchanged. Since these alterations in atrial ANF concentration are similar to findings in other conditions exhibiting high circulating levels of ANF, e.g., the spontaneously hypertensive rat, the deoxycorticosterone acetate–salt hypertensive rat, it seems unlikely that the genetic defect of the cardiomyopathic hamster is specifically responsible for changes in ANF synthesis, storage, or release.

To determine the extent of changes in cardiac ANF...
content and biosynthesis in heart failure and to determine whether ventricular tissue contributed significantly to this disease-induced change, immunoreactive ANF was measured in plasma and atrial and ventricular tissues of cardiomyopathic and control hamsters. Additionally, ANF mRNA was determined in both atrial and ventricular tissue of diseased and normal hamsters.

Materials and Methods

Male cardiomyopathic (strain Bio 14.6) and outbred golden Syrian (control) hamsters were obtained from Canadian Hybrid Farms, Kings County, Nova Scotia. Radioisotopes and nick translation materials were purchased from Amersham, Arlington Heights, Ill. Klenow fragment of DNA polymerase I and Hind III digested λ-DNA were purchased from New England Biolabs, Beverly, Mass. Rabbit anti-rat ANF antisera was obtained from Peninsula Laboratories, Belmont, Calif. Synthetic rat ANF (Arg10-Tyr39) was provided by Dr. Stephen Brady, Merck Sharp & Dohme Research Laboratories, West Point, Penn. The agarose bound goat anti-rabbit IgG antibody was obtained from Miles Laboratories, Naperville, Ill. The plasmid pANF 3A containing rat ANF complementary DNA (cDNA) and the plasmid containing creatinine kinase cDNA were previously described.

Classification

Three groups of hamsters, 290–350 days old, were studied: controls, cardiomyopathic with moderate heart failure, and cardiomyopathic with severe heart failure. Myopathic animals were considered in severe failure if they showed increased liver or lung weights per 100 g body weight (>2 standard deviations above the mean of animals in moderate failure), gross lesions of liver congestion, or atrial thrombi and atrial weights of >70 mg/100 g body weight (>2 standard deviations above the mean of animals in moderate failure if they showed increased liver or lung weights per 100 g body weight). The remaining myopathic animals were considered in moderate failure. In a subset of hamsters used in the present study, central venous pressure was 0.2 ± 0.1 cm of water in control animals and was elevated to 0.8 ± 0.3 and 3.4 ± 0.9 cm of water in hamsters in moderate and severe heart failure, respectively.

Tissue and Plasma ANF Levels

Methods for assessing ANF tissue and plasma levels have been previously described in greater detail. In brief, 2 ml of blood was withdrawn from the aorta under sodium pentobarbital anesthesia (55 mg/kg i.p.) into syringes preloaded with 3 mg EDTA, 150 μg aprotinin, and 50 μg soybean trypsin inhibitor. Plasma was acidified and then extracted on a Sep-Pak C18 cartridge and eluted with a solution consisting of 86% ethanol, 4% acetic acid, and 10% distilled water. The right and left atria for each animal were combined, and a 50-mg piece of the apex formed the ventricular sample. The tissue was extracted in 0.1N acetic acid with 1 μg/ml pepstatin A, 1 μg/ml PMSF, and 1,200 counts/min [125I]rat-ANF 28 (Amersham) added and placed at 100°C for 10 minutes. Percent recovery was measured by counting 1 ml of extract in a gamma counter for 10 minutes. All tissue radioimmunoassay (RIA) results were corrected for recovery. RIA was performed with Peninsula primary antibody, rat-ANF as standard, and [125I]rat-ANF 28 as label. Separation was achieved using a solid phase anti-IgG antibody.

RNA Isolation and Blot Analysis

Total cellular RNA was isolated from atria and ventricular samples by the guanidine isothiocyanate-CsCl procedure. Ten micrograms of ventricular and 5 μg of atrial RNA (as determined by ultraviolet absorption at 260 nm) were electrophoresed on 1.2% agarose gels containing formaldehyde, and DNA was electrophoresed as a molecular weight standard. The RNA was transferred by the method of Thomas and baked at 80°C for 2 hours. Completeness of transfer was determined by staining the gel after transfer with ethidium bromide and viewing under ultraviolet illumination. The filters were prehybridized in 50 mM NaPO4, pH 6.8; 0.1% NaDodSO4; 0.75 M NaCl; 0.075 M Na citrate, pH 7.0; 50 μg/ml polyadenylate; 50 μg/ml salmon sperm DNA; 0.2% bovine serum albumin; 0.2% Ficoll; and 0.2% polyvinyl-pyrollidone at 54°C for 2 hours. pANF 3A was labeled with 32P by nick translation, and 1 × 106 counts/min were added and the hybridization continued for 24 hours at 65°C. The filters were washed (4 times for 30 minutes) in 0.15 M NaCl, 0.015 M Na citrate, and 0.1% NaDodSO4 at 65°C, air dried, and autoradiographed. The autoradiographic signals were quantitated by a scanning densitometer (Hoeffer Instruments, San Francisco) and the values normalized so that control ventricle or atria was one unit. Filters were rehybridized with a 32P-labeled plasmid containing the gene for creatinine kinase after washing in 15 mM NaCl, 1.5 mM Na citrate, and 0.1% NaDodSO4 (4 times) heated to 95°C and allowed to cool to 65°C, and processed as described above.

Statistical analysis of RNA data was accomplished using Jonckheere's test for equality against ordered alternatives. A ranking, nonparametric evaluation was used because a normal distribution could not be assumed for this data set.

Results

The plasma, atrial, and ventricular ANF titration curves were parallel to the ANF standard curve and to curves constructed from titration of atrial and plasma extracts (Figure 1). These data indicate that the extracts contained an antigen peptide indistinguishable from synthetic rat ANF in its antibody binding characteristics. Dilution curves constructed for the RIA data from cardiomyopathic hamster atria, ventricles, and plasma extract were also parallel to the standard curve (data not shown).

In agreement with our previous results, plasma concentration of ANF was 5 times higher in hamsters in moderate heart failure when compared with control hamsters, increasing to 14 times higher in animals with severe heart failure (see Table 1). In contrast, atrial
Franch et al  Ventricular Production of ANF in Heart Failure

Plasma samples were concentrated fourfold. Extracts were assayed using Peninsula primary antibody, rat-ANF as standard and [125I]rat-ANF 28 as label. All dilution curves were parallel (ANOVA, p<0.05).

In contrast to the atrial ANF, ventricular ANF concentration was greater in cardiomyopathic than control hamsters. ANF concentration in ventricular samples (Figure 2B) was elevated threefold and sevenfold in moderate and severe failure, respectively. Unlike the atria, ventricular mass was not significantly increased in this dilated cardiomyopathy; total ventricular ANF content (ANF concentration x ventricular weight) was increased proportionally with ventricular ANF concentration (Table 1). Ventricular ANF concentration as a percentage of atrial ANF concentration was almost 50 times higher in animals with severe failure compared with controls (11.2 ± 2.7% compared with 0.24 ± 0.05%). As a consequence, the ventricle contributed a significant proportion of total cardiac ANF stores in cardiomyopathic animals, representing 9% and 23% of cardiac ANF content in moderate and severe heart failure, respectively, as opposed to 3% in control hamsters.

Atrial Natriuretic Factor mRNA Analysis

To determine if the changes in atrial and ventricular concentration of ANF reflect changes in the underlying gene expression, mRNA from the atria and ventricles was analyzed. The hamsters used to provide the mRNA were well matched with the hamsters used in the previous study. Their plasma ANF levels were similar (50 ± 4, 478 ± 78, and 1,105 ± 185 pg/ml for control hamsters and hamsters in moderate and severe heart failure, respectively), while their ventricular ANF concentrations were slightly lower (28 ± 6, 95 ± 13, 159 ± 34 ng/100 mg ventricular wt). Using a rat ANF cDNA clone as a probe, a single major band of ~900 nucleotides corresponding to the size of the ANF mRNA 5'-3' was observed in RNA from all animals in both atrial and ventricular tissues (Figure 3). While consistent strong hybridization was observed for all atrial RNA, hybridization to the control ventricle RNA was faint. In contrast, cardiomyopathic ventricular RNA contained increased amounts of ANF mRNA relative to the control ventricle RNA. Two faint, high molecular weight bands were also seen in cardiomyopathic atria and ventricles.

Hamsters with moderate heart failure had 7 times, and hamsters with severe heart failure 13 times, more ventricular ANF mRNA than control ventricles, while there was no significant difference between atrial ANF mRNA concentrations (Figure 4). The magnitude of the increase in ventricular mRNA was greater than the observed variability, indicating a major difference in the amount of ANF mRNA expressed in the ventricles of heart failure animals compared with control. To determine if the change in amount of ANF mRNA was specific or reflected changes in all messages, the level of creatine kinase mRNA was examined. Creatine kinase mRNA was found to increase twofold in the atria and decrease 50% in the ventricles of severe animals relative to control animals. These differences were not

Table 1. Atrial Natriuretic Factor Concentration or Content in Plasma, Atria, and Ventricles of Cardiomyopathic Hamsters

<table>
<thead>
<tr>
<th>Classification</th>
<th>Body weight (g)</th>
<th>Atrial weight (mg)</th>
<th>Ventricular weight (mg)</th>
<th>Plasma ANF concentration (pg/ml)</th>
<th>Total atrial ANF content (µg)</th>
<th>Total ventricular ANF content (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=11)</td>
<td>118 ± 5</td>
<td>27 ± 1</td>
<td>423 ± 12</td>
<td>90 ± 17</td>
<td>5.08 ± 0.52</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>Moderate failure (n=10)</td>
<td>107 ± 4</td>
<td>51 ± 6*</td>
<td>389 ± 10</td>
<td>485 ± 154*</td>
<td>4.91 ± 0.87</td>
<td>0.50 ± 0.05*</td>
</tr>
<tr>
<td>Severe failure (n=11)</td>
<td>116 ± 2</td>
<td>126 ± 7†</td>
<td>438 ± 11</td>
<td>1,299 ± 391†</td>
<td>3.94 ± 0.50</td>
<td>1.19 ± 0.13†</td>
</tr>
</tbody>
</table>

Classification of animals by severity of heart failure is described in text. Control animals were outbred golden Syrian hamsters. All data are presented as mean ± SEM, and results were analyzed for statistical significance with Dunnett's Multiple Range test.

*Significantly different from control (p<0.05).
†Significantly different from control and moderate failure (p<0.05).
Discussion

Cardiac ANF biosynthesis was not decreased in the cardiomyopathic hamster. As noted previously, while the atrial concentration of ANF (per milligram of tissue) declined, atrial weight was increased so that the total atrial content (i.e., atrial ANF per hamster) remained unchanged. The smaller atrial ANF concentration in cardiomyopathic animals was not due to decreased gene expression since atrial ANF mRNA

significant. Thus, the dramatic increase in ANF mRNA observed in severely myopathic ventricles was probably specific and independent of overall mRNA synthesis. It should be noted that the total amount of poly A+ RNA that is recovered does not vary significantly between control and myopathic animals (H.A. Franch and R.A.F. Dixon, unpublished observations).

FIGURE 2. Atrial (top) and ventricular (bottom) tissue concentrations of ANF in control hamsters (C) and myopathic hamsters with moderate (M) or severe (S) cardiac failure. ANF concentrations were measured by RIA as described in "Materials and Methods." Open symbols represent mean±SEM.

FIGURE 3. Expression of ANF mRNA in atrial (A) and ventricular (V) tissue from control (C) and myopathic hamsters with moderate (M) or severe (S) cardiac failure. This is a representative autoradiograph in which 10 μg of ventricular and 5 μg of atrial ANF was electrophoresed on agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized with "P-labeled rat ANF probe. Molecular weight was determined using Hind III digest of phage λ-DNA labeled with "P with Klenow fragment of DNA polymerase I . ANF band is at ~900 nucleotides.

FIGURE 4. ANF mRNA levels in atria (open bars) and ventricle (hatched bars) from control (n=4,6) and myopathic hamsters with moderate (n=4,5) or severe (n=6,6) cardiac failure. Quantitation of autoradiograph was accomplished using a scanning densitometer and adjusted for sample size (i.e., atrial results were doubled). Data are presented as mean±SEM. *Significantly different from control (p<0.05).
diets and in chronic high ANF states, such as the spontaneously hypertensive rats where atrial weight is unchanged. Thus, changes in granularity observed in atria from cardiomyopathic hamsters are consistent with the decrease in [atrial ANF]/mg tissue (Figure 2A), but no change in atrial content (atrial ANF)/hamster or ANF mRNA indicated that total ANF biosynthesis was probably unchanged in the atria of these animals.

The most striking finding of this study was that the ventricles of myopathic hamsters contained 23% of the total cardiac ANF compared with 3% in control animals. The increased abundance of ventricular ANF mRNA in hamsters with heart failure suggests a concomitant increase in ANF peptide synthesis. Increases in ventricular ANF may not be limited to heart failure but may contribute to other high circulating ANF states. Ventricular mRNA is also increased in several models of volume overload and in spontaneously hypertensive rats (R.A.F. Dixon, unpublished observations).

Although ANF-containing granules have been observed in ventricles of nonmammalian animals, similar granules have not been observed in adult or fetal ventricular cardiocytes of mammals. Large quantities of ANF have not been detected in mammalian ventricles and normal rat ventricular extracts were not active in causing natriuresis and diuresis. The lack of granules may be due to differential processing of ANF. Matsuo found 31 ng ANF/100 mg ventricular wt in normal rat ventricles; this close to the 39 ng ANF/100 mg ventricular wt we found in normal hamsters. This amount of ANF is too small to elicit natriuresis and diuresis in most bioassay systems. The first study on the molecular form of ANF in the ventricle indicates that it is primarily the pro-hormone. However, unlike the atria, the low molecular weight circulating form is also seen. Whether this is an artifact of extraction or a result of differential processing remains to be determined. The ANF message in the ventricle appears to be the same size as in the atria.

The ANF gene is present as a single copy in the genome and is probably being expressed in normal atria near its maximum rate, with its message representing 1 to 3% of total mRNA. The largest reported increase in ANF mRNA was twofold in volume expanded rats. It is possible that the slight increase in atrial mRNA seen in our study reflects a change of similar magnitude. If the atria are maximally producing ANF, greater demand for ANF could be met by degranulation. The ventricle could be recruited for production of ANF in situations where there is a long-term, high demand for ANF. The trigger for the increased production of ANF in the ventricle is unknown. The ventricular dilation of hamster cardiomyopathy is marked by increased ventricular pressures, increased wall stress, and decreased cardiac compliance. As suggested by Lattion et al., an increase in wall stress could be a signal for increased production of ANF in ventricles, as it likely is in atria.

In summary, elevated concentrations of immunoreactive ANF in ventricular tissue and plasma and increased ventricular ANF mRNA levels were associated with the degree of severity of heart failure in cardiomyopathic hamsters. Since similar changes were not observed in atrial tissues, we propose that there are differences between atria and ventricles in the synthesis and/or storage of ANF and in heart failure, the ventricle is potentially an important source for this hormone in this disease state.

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