Transcription, storage and release of atrial natriuretic factor in the failing human heart

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SUMMARY

1. In this study the relationship between the synthesis of atrial natriuretic factor at the level of atrial natriuretic factor mRNA and the atrial storage and circulating plasma levels of atrial natriuretic factor were investigated in 15 patients with heart failure. The patients underwent right and left heart catheterization before cardiac surgery for valve replacement or coronary artery bypass grafting.

2. Plasma concentrations of atrial natriuretic factor were correlated to atrial levels of atrial natriuretic factor mRNA. Atrial levels of atrial natriuretic factor mRNA and plasma concentrations of atrial natriuretic factor exhibited a close correlation to both pulmonary artery pressure and left atrial pressure. No relationship, however, could be found between the right atrial content of atrial natriuretic factor and both the expression of atrial natriuretic factor mRNA in the atria and the plasma levels of atrial natriuretic factor.

3. From these data it may be concluded that increased plasma levels of atrial natriuretic factor in the pressure- and/or volume-overloaded heart are associated with an elevated level of atrial natriuretic factor mRNA. We suggest that not only plasma levels of atrial natriuretic factor but also the expression of atrial natriuretic factor mRNA in the atria and the plasma levels of atrial natriuretic factor.

INTRODUCTION

The atrial natriuretic factors (ANFs) represent a family of peptides with potent natriuretic, diuretic and vasorelaxant properties. The precursor peptide (prepro-ANF, with 151 amino acids) is synthesized and stored in secretory granules of cardiac myocytes (for reviews, see [1-3]). Upon secretion the circulating active forms are processed by enzymic cleavage [4].

The structure of the gene encoding for prepro-ANF and the nucleotide sequence of the ANF mRNA have been published [5-7]. The successful cloning of prepro-ANF complementary DNA (cDNA) has led to several experimental studies emphasizing the role of ventricular activation in the secretion of ANF into the circulation under conditions of left ventricular pressure and/or volume overload [8-10]. In a rat model of myocardial infarction the atrial content of ANF mRNA was found to be elevated with increasing infarct and atrial size, and atrial storage of ANF was shown to be diminished due to cardiac failure [11].

In congestive heart failure, independent of its nature and duration, increased plasma concentrations of ANF have been reported and were closely related to cardiac filling pressures [12-14]. Further studies indicated that atrial distension was the predominant stimulus for the release of ANF into the circulation in man [15, 16]. Even during chronically elevated atrial distension, the release of ANF both at rest and during exercise was unaltered [17].

So far, little is known about the factors contributing to the maintenance of chronically elevated plasma levels of ANF in the failing human heart.

The present study was therefore designed to examine the regulation of the synthesis of ANF mRNA and to elucidate the relationship between synthesis, storage and circulating plasma levels of ANF in human congestive heart failure.

MATERIALS AND METHODS

Characteristics of patients

The study population consisted of 15 patients (eight men and seven women) with heart failure (New York Heart Association II-III; mean age 57.5 ± 3.3 years, range
29–78 years). The underlying heart diseases were: aortic valve disease (nine patients), mitral valve disease (two), mixed left-sided valvular heart disease (two) and coronary artery disease (two). The patients were on long-term medications consisting of digitalis \((n=10)\), nitrates \((n=6)\), diuretics \((n=12)\), calcium-channel blockers \((n=6)\), angiotensin-converting enzyme inhibitors \((n=3)\), quinidine \((n=1)\) and flecainide \((n=1)\). These medications remained unchanged during the study. After assessment of the severity of the underlying heart disease by cardiac catheterization, the patients underwent surgery for either valve replacement \((n=13)\) or coronary artery bypass grafting \((n=2)\). The mean interval between cardiac catheterization and cardiac surgery was 9.5 ± 2.5 weeks. Only patients with plasma creatinine levels within the normal range \((<1.2 \text{ mg/dl})\) were included in the study. The study was approved by the ethical committee of the University of Heidelberg.

### Cardiac catheterization

After oral premedication with 10 mg of diazepam, right and left heart cardiac catheterization was performed using the Seldinger technique from the right femoral vein and artery. Pulmonary artery, right atrial, left atrial (after trans-septal puncture), left ventricular and arterial blood pressures were recorded. In patients with coronary artery disease, in whom the left atrium was not catheterized \((n=2)\), pulmonary capillary wedge pressure served as an indicator of left atrial pressure. In all patients coronary angiography was performed using the Judkins technique.

### Determination of ANF in the peripheral blood

The day before cardiac surgery, 10 ml of blood was drawn for the determination of plasma ANF concentrations. All samples were immediately placed into pre-chilled heparinized tubes. Cellular elements were separated by centrifugation \((5000 \text{ rev./min, 15 min, } 4°C)\) and the plasma was frozen immediately and stored at −30°C. For r.i.a. the plasma was extracted with C₁₉-Sep-Pak cartridges (Waters Associates Inc., Milford, MA, U.S.A.), as described previously [18]. The recovery from this extraction procedure was 70%. R.i.a. was performed using our own antiserum at a final concentration of \(1/120000\) [16]. The sensitivity was 2.5 fmol/tube. The inter- and intra-assay variations averaged 10 and 5%, respectively. Our antiserum shows a complete cross-reactivity with human ANF \((99–126)\) as well as with rat ANF \((99–126)\), but not with rat ANF \((103–126)\). All plasma levels were corrected for 70% recovery.

### Tissue preparation

Samples of atrial tissue were obtained from the apex of the right auricle, when venous canulae were inserted into the right atrium for initiation of extracorporal circulation. The tissue samples \((\text{mean wet weight } 89 ± 5.8 \text{ mg})\) were immediately frozen in liquid nitrogen and stored at −70°C.

### Extraction of ANF from atrial tissue

Frozen atrial tissue was divided, weighed, dissolved in 2 ml of 0.1 mmol/l HCl and heated in a boiling water bath for 15 min. Then the samples were homogenized, followed by centrifugation at 20,000 g for 30 min at 4°C. The supernatants were transferred into polypropylene tubes and evaporated to dryness in a Speed Vac concentrator. The samples were then reconstituted in the standard r.i.a. buffer \([0.1 \text{ mol/l Tris, pH 7.4, containing } 0.1\% \text{ (w/v) gelatin} ]\), diluted to an adequate concentration and then subjected to r.i.a. as described above.

### Extraction of total RNA and Northern blotting

Total RNA was extracted by the LiCl/urea technique [19] and quantified by measuring the absorbance at 260 nm.

A predetermined amount of RNA \((2.5 \mu\text{g})\) was de-naturated in glyoxal buffer \([1 \text{ mol/l glyoxal, } 50\% \text{ (w/v) dimethylsulphoxide, } 10 \text{ mmol/l sodium dihydrogen phosphate, pH 7.0}]\) loaded on to a 1.2% \((\text{w/v})\) agarose gel and fractionated electrophoretically \((4 \text{ h, } 50–100 \text{ V})\). The gel was then transferred to a Nytran 13N membrane. For preparation of an ANF-specific cRNA probe, a 580 bp PstI-restricted prepro-ANF cDNA fragment (obtained from K. Bloch and J. Seidmann, Boston, MA, U.S.A.) was subcloned in a pSP64 plasmid [5]. The EcoRI-linearized plasmid served as a template for ANF cRNA transcription using 100 µCi of \([\alpha-32P]\)UTP \((400 \text{ Ci/mmol; Amersham, Braunschweig, F.R.G.})\) and the SP6 polymerase promoter system described by Melton et al. [20].

Hybridization was achieved in 50% \((\text{w/v})\) formamide, 50 mmol/l sodium phosphate, pH 6.8, 1 mol/l sodium chloride, 200 µg/ml herring sperm DNA, \(5 \times \text{ Denhardt's solution, } 0.1\% \text{ (w/v) SDS and } 10 \text{ mmol/l EDTA (sodium salt) for } 18 \text{ h at } 65°C\). The membranes were washed in \(2 \times \text{ SSC (}1 \times \text{ SSC is } 0.15 \text{ mol/l NaCl/0.015 mol/l trisodium citrate)/0.1\% (w/v) SDS at room temperature twice for 5 min followed by washing in } 1 \times \text{ SSC/0.1\% (w/v) SDS for } 15 \text{ min at } 65°C\). The last washing step was performed in 0.1 \(\times \text{ SSC/0.1\% (w/v) SDS at } 65°C \text{ for } 15 \text{ min and the membrane was then exposed to an X-ray film at } −70°C\).

The specific bands of ANF mRNA were cut out and the radioactivity of each sample was measured in a β-scintillation counter. The level of ANF mRNA was determined as the ratio of the level of ANF mRNA in the sample to the level of ANF mRNA in the standard. This internal standard consisted of pooled tissue derived from human right auricles. A fraction of this pool tissue was extracted in parallel with the samples and blotted in different concentrations (see Fig. 1). The ANF mRNA content of 2.5 µg of standard tissue represented 1 unit.

In order to avoid unspecific influences affecting the quantification of ANF mRNA, such as pressure-induced atrial hypertrophy and the consequent increase in total atrial protein synthesis, the ratio of ANF mRNA to β-actin mRNA was determined in seven patients (nos. 1–7).

For the preparation of a β-actin-specific cRNA hybridization probe, a 640 bp SalI–EcoRI fragment of a β-actin-like pseudogene was subcloned into a pGEM4 plasmid [21]. The HindIII-linearized plasmid served as
template for transcription with the SP6 polymerase system as described for ANF.

**Statistical analysis**

Data are expressed as means ± SEM. Linear regressions were obtained by the method of least squares. Intra-group differences (body weight, plasma sodium concentration, packed cell volume) were tested by paired t-test and Wilcoxon matched pairs test. A P value of less than 0.05 was considered statistically significant.

**RESULTS**

The characteristics of the patients are summarized in Table 1.

**Haemodynamic measurements**

During cardiac catheterization the following haemodynamic parameters were assessed: mean right atrial pressure (6.7 ± 0.6 mmHg, range 3–11 mmHg, n = 15), mean left atrial pressure (11.6 ± 0.8 mmHg, range 7–15 mmHg, n = 14), mean pulmonary artery pressure (19.5 ± 1.1 mmHg, range 14–30 mmHg, n = 15), mean arterial pressure (89.6 ± 3.0 mmHg, range 75–105 mmHg, n = 15) and cardiac index (2.73 ± 0.1 L min⁻¹ m⁻², range 2.0–3.31 L min⁻¹ m⁻², n = 14).

**Plasma ANF concentrations**

Plasma ANF levels (97.1 ± 26.4 pg/ml, range 27.4–436.9 pg/ml, n = 15), determined by RIA of venous blood samples on the day before cardiac surgery, were found to be correlated to mean pulmonary artery pressure (r = 0.65, P < 0.01, n = 15) and mean left atrial pressure (r = 0.60, P < 0.05, n = 14). No significant relationship was observed between plasma ANF concentrations and mean right atrial pressure (r = -0.074, not significant, n = 15) in this patient cohort.

**Tissue ANF concentrations**

Tissue ANF levels in the right heart auricle (183.9 ± 49.3 pg/g wet tissue, range 40.3–789.3 pg/g wet tissue, n = 15) were found to be independent of cardiac filling pressure, plasma ANF concentration and right auricle ANF mRNA level. A tendency towards lower tissue ANF concentrations in patients with a reduced cardiac index was observed (r = -0.46, P < 0.1, n = 14).

**Northern blotting of ANF mRNA**

Fig. 1 shows the results of the Northern blotting procedure for determination of the level of ANF mRNA in the 15 patients studied. The first five bands represent the five different concentrations of the internal standard.

A significant correlation was found between the level of atrial ANF mRNA and plasma ANF concentration (r = 0.65, P < 0.01, n = 15). The level of ANF mRNA was also related to mean left atrial pressure (r = 0.68, P < 0.01, n = 14, Fig. 2) and mean pulmonary artery pressure (r = 0.78, P < 0.001, n = 15, Fig. 3). No relationship was observed between the level of ANF mRNA and both the tissue concentration of ANF (r = 0.23, not significant, n = 15) and the right atrial pressure (r = 0.18, not significant, n = 15).

Table 2 summarizes the expression of ANF mRNA and β-actin mRNA in the right heart auricle of the patients studied. The atrial ANF mRNA/atrial β-actin mRNA ratio increased with left ventricular filling pressure (r = 0.91, P < 0.01 and r = 0.80, P < 0.05, respectively, Figs. 4a and 4b), indicating that the observed relationship between atrial ANF mRNA level and left ventricular overload was not caused by a non-specific increase in overall peptide biosynthesis.

**DISCUSSION**

In congestive heart failure chronically elevated plasma concentrations of ANF have been observed [13, 14] and were shown to be independent of the nature and duration of the underlying heart disease [17]. The successful cloning of prepro-ANF cDNA has led to a number of experimental studies focusing on the role of ventricular activation of ANF mRNA synthesis in experimental heart failure [8, 9, 22].

Despite ventricular recruitment the majority of the circulating peptide is released by atrial myocytes. The ANF mRNA expressed in both atria represents 3% of all atrial mRNA species [4]. In the present study a correlation between left atrial and pulmonary artery pressures and the ANF mRNA level in the right auricle was observed. Our data suggest that increased left ventricular filling pressure induces an enhancement of the atrial level of ANF mRNA in chronic heart failure. Hence, atrial distension is the predominant stimulus not only for the release of the peptide [18, 23], but also for the elevation of the intra-atrial level of ANF mRNA in response to pressure and/or volume overload in man. This is in agreement with experimental studies in rats with severe left ventricular dysfunction after myocardial infarction [11, 24]. In the present study a linear relationship between ANF mRNA level and right atrial pressure could not be demonstrated. This is in contrast to the observed relationship between ANF mRNA and left cardiac filling pressure. The most likely explanation for this observation is the very small range and sensitivity of the measurement of right atrial pressure in our patients.

An increased release of ANF, indicated by elevated plasma ANF concentrations, was combined with an activation of ANF mRNA transcription, which may prevent the failing heart from becoming depleted of its ANF tissue stores. The increase in ANF mRNA level was not accompanied by a simultaneous increase in β-actin expression, indicating a specific effect of elevated left ventricular filling pressure on the level of ANF mRNA and not a non-specific increase in total atrial protein synthesis.
Table 1. Characteristics of patients studied

Abbreviations: ANF mRNA, ratio of ANF mRNA level in sample to ANF mRNA level in standard; plasma ANF, venous plasma concentration of ANF; AR, aortic regurgitation; AS, aortic stenosis; CAD, coronary artery disease; CI, cardiac index; CC, at time of cardiac catheterization; CS, at time of valve replacement/coronary artery bypass grafting; LAP, mean left atrial pressure; MAP, mean artery pressure; MS, mitral stenosis; MVD, mixed left-sided valvular heart disease; PAP, mean pulmonary artery pressure; RAP mean right atrial pressure.

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<th>Tissue ANF (μg/g)</th>
<th>ANF mRNA (l/min -1 m-2)</th>
<th>β-Actin mRNA (c.p.m.)</th>
<th>CI (l/min)</th>
<th>MAP (mmHg)</th>
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SEM 3.3

*Pulmonary capillary wedge pressure.
Heart catheterization with haemodynamic measurements was performed several weeks before surgery when atrial tissue and plasma samples were obtained for determination of ANF mRNA and plasma ANF concentrations. Although an influence of this time interval cannot be ruled out, it is not likely that the haemodynamic profile changed markedly, as the medication remained constant and there was no evidence of major volume shifts, since body weight, plasma sodium concentration and packed cell volume did not show a directional change in this time interval.

The atrial levels of ANF mRNA and the plasma ANF concentrations were linearly related. This relationship might reflect augmented ANF mRNA synthesis for the maintenance of elevated plasma ANF concentration and increased ANF secretion in congestive heart failure. On the other hand, a decreased breakdown of ANF gene transcriptional fragments cannot be excluded. In this way an altered post-transcriptional modification of the primary gene transcript could also be responsible for the observed increased ANF mRNA level under conditions of constant transcription.

No evidence exists that atrial synthesis of ANF mRNA was down-regulated by high levels of the circulating hormone, as applies to peripheral ANF-binding sites in human platelets [25]. Since no down-regulation could be observed, the close relationship between plasma ANF concentration and atrial level of ANF mRNA might reflect a modulation of both parameters by atrial distension.
Atrial levels of ANF mRNA and release of ANF were activated by pressure and/or volume overload in man and may influence tissue stores of ANF, which reflect the enhanced synthesis of ANF mRNA and the increased release of the peptide. In accordance with previous studies in man [26], no relationship between plasma and atrial tissue concentrations of ANF was observed. Recently, no significant correlation between right atrial \( \beta \)-ANF and both pulmonary artery pressure and pulmonary capillary wedge pressure in patients with aortic valve disease and an atrial septal defect could be demonstrated [27]. In our patients an elevated level of atrial ANF mRNA and a concomitant increase in plasma ANF concentration did not result in a significant change in tissue ANF concentration. These data suggest that the increased atrial level of ANF mRNA compensates for the elevated plasma level of ANF without markedly affecting the tissue level of ANF.

In summary, the results of the present study indicate that the human failing heart is not ANF-depleted, since the ability of the failing heart to maintain a chronically elevated plasma concentration of ANF, with its beneficial effects on left ventricular function, seems to be unaltered. Our data suggest that increased left ventricular filling pressure causes long-term activation of the ANF system to counterbalance the stimulated vasopressor systems in congestive heart failure.

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