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Molecular Weight Heterogeneity of Plasma-ANF in Cardiovascular Disease

R. M. Arendt¹, A. L. Gerbes¹, D. Ritter², and E. Stangl¹

¹ Medizinische Kliniken I und II, Ludwig-Maximilians-Universität, Klinikum Großhadern, München

² Abteilung Neuropharmakologie, Max-Planck-Institut für Psychiatrie, Martinsried

Summary. Structural differences of circulating ANF may partly explain why the physiological response of the heart in controlling volume/pressure loading in cardiovascular disease states remains insufficient in spite of elevated ANF plasma levels. Structural analysis of plasma ANF immunoreactivity was performed by means of gel permeation of plasma extracts subsequent to radioimmunoassay. ANF plasma levels in hypertensive patients or patients with congestive heart failure (CHF) were significantly elevated as compared to normotensive controls or cirrhotics. (61.7 ± 13.2 or 81.5 ± 32.7 versus 9.6 ± 1.0 or 10.3 ± 1.3 fmol/ml, $p < 0.01$). In CHF patients, ANF plasma concentrations were significantly correlated to right atrial and pulmonary capillary wedge pressures. ANF release was stimulated by head-out water immersion both in normotensive controls and cirrhotic patients. No higher molecular weight forms were detected in plasma of control subjects. 15000-dalton ANF, in addition to 3080-dalton ANF, was present in plasma of hypertensive patients and, in trace amounts, of cirrhotic patients. In some CHF patients, elevated ANF plasma levels predominantly comprised higher molecular weight forms of approx. 15000 daltons MW, in addition to considerable amounts of ANF immunoreactivity presumably bound to larger proteins that eluted in the void volume. The data suggest that a dysregulation of post-translational processing of ANF may contribute to the pathophysiology of cardiovascular disease.

Key words: ANF plasma levels – Chromatographic analysis – Processing – Cardiovascular disease – Cirrhosis

mehrten Ausscheidung von Wasser und Natrium, einer Tendenz zu verstärkter Auswärtsfiltration im Kapillarbett und wahrscheinlich zu einer Verringerung des Durstgefühls (An increase in intrathoracic blood volume leads, via endocrine mechanisms, to an enhancement of water and sodium excretion, an increase in outward filtration in the capillary bed and, probably, to a decreased thirst sensation)” [1]. Gauer’s endocrine mechanisms may very well be mediated by the recently discovered Atrial Natriuretic Factor (ANF) [2, 2a]. This has emerged as an excellent candidate for the long elusive “Third Factor”, the postulated natriuretic hormone [3] that, besides vasopressin and the renin-angiotensin-aldosterone system, governs sodium excretion and thereby helps to regulate extracellular fluid volume and blood pressure. Since it is also a potent vasodilator [4, 5], ANF may be the heart’s “own” hormone that, by reduction of pre- and afterload, participates in defining optimal conditions for efficient cardiac performance.

The heart’s smallest endocrine unit is the atrial myoendocrine cell [6], the biological function of which is determined by 1) biosynthesis of the prohormone, 2) storage of the prohormone in specific granules, and, upon the appropriate stimulus, 3) secretion of the biologically active material by exocytosis. Under normal conditions the post-translational processing of the prohormone is likely to be coupled to the secretion stimulus and may occur at the site of secretion. We have recently demonstrated the presence of the processing product ANF-28 in the circulation of normotensive subjects [7]. Surprisingly, it has also been shown that patients with cardiovascular disease characterized by volume/pressure loading have considerably higher plasma levels of ANF than normotensive counterparts [7–10]. Cirrhotic patients

“Das vergrößerte intrathorakale Blutvolumen führt über endokrine Mechanismen zu einer ver-

showing an increased extracellular volume, though mainly confined to an extravascular sub-compartment, also tended to have increased plasma levels, though not in all studies significantly so [11–13]. To study the overall stimulus-response-coupling we utilized Gauer's head-out water immersion experiment [14], an important investigative tool in the examination of the secretory function of the heart [15, 16]. In addition to alterations in stimulus-response-coupling, a defective post-translational processing of pro-ANF (ANF-126) or a modified target tissue responsiveness to circulating ANF might be important features in the pathophysiology of volume homeostasis. We have recently demonstrated that the molecular weight pattern of plasma-ANF in hypertensive patients differs from that seen in normotensive controls [8]. Elevated ANF plasma levels in patients with congestive heart failure were predominantly constituted by such higher molecular weight forms not apparent in normal subjects [17]. In this article, we document plasma levels of ANF in hypertensive patients, patients with congestive heart failure before and under converting enzyme inhibitor therapy, and in normotensive subjects and cirrhotic patients prior to and during head-out water immersion. An initial structural analysis of circulating ANF was achieved by use of high performance gel permeation chromatography of plasma extracts from these patients.

Methods

Extraction of plasma samples and RIA procedures were modified from [7]. Briefly, antibody Toni III was substituted for the less sensitive antibody Toni II. This antibody is mid-molecule- and C-terminal directed. Cross reactivity with the N-terminally extended cardiodilatin-88 (gift from Prof. WG Forssmann, Heidelberg, FRG) was 29.7%, 35.8% to rat ANF-28, 13% to atriopeptin III, 0.03% to atriopeptin I or II. It did not cross-react with a wide variety of peptides and proteins, including its immunization conjugate (bovine thyroglobulin). The final titer was 1:120 000 and the assay sensitivity was 0.5 fmol/assay tube. The 50%-binding intercept of the standard curve was 7 fmol. Synthetic standards and iodinated labels were from NovaBiochem, Läufelfingen, Switzerland. 0.5 to 3 ml-plasma aliquots were extracted by adsorption to pre-rinsed Amberlite XAD-2 adsorbent resin (particle size 0.3–1.0 mm, Serva, Heidelberg, FRG) [7]. Recovery of synthetic ANF-28 was approximately 67%. Levels were not yet corrected for recovery (the results of the "International Col-

laborative Study Of The Proposed International Standard For Atrial Natriuretic Factor On Behalf Of The AHA/ISH/WHO" pending). The intra-assay coefficient of variation ($n=6$) was $< 5\%$. 5 ml plasma extracts were subjected to high performance gel permeation chromatography on a 7.5×600 mm TSK-125 Bio Sil column (Bio Rad, Munich, FRG), eluted with 0.09% TFA containing 0.005 M Na_2SO_4 plus 0.002 M NaH_2PO_4 and 30% acetonitrile as a solvent. Flow rate was 0.4 ml/min, and aliquots from column fractions were analyzed for immunoreactive(ir)-ANF. Peripheral blood was drawn into pre-cooled syringes and immediately transferred to pre-cooled polystyrene tubes containing 500 kallikrein inhibitor units/ml aprotinin and 1 mg/ml sodium EDTA. Plasma was separated and stored at -70°C until extraction. 50 normotensive control subjects showing no evidence of cardiovascular, renal, pulmonary or gastrointestinal disease took part in the study. 36 patients with essential hypertension were examined also; at the time of examination their mean blood pressure was 171 ± 4 over 100 ± 3 mm Hg. In addition, 17 patients with congestive heart failure, functional class NYHA II–IV, were studied. Patients were hospitalized 1 week before catheterization of the heart and all medication was discontinued except for diuretics and digitalis. Measurements were taken before, immediately following and 6 months after institution of therapy with an angiotensin converting enzyme inhibitor (enalapril, usually 2×5 mg bid). 31 patients with cirrhosis of the liver, confirmed by biochemical and histological examination, were investigated. Cirrhotic patients were divided into subgroups with and without ascites. 12 healthy controls and 11 cirrhotic patients were subjected to head-out water immersion procedures. After voiding, subjects assumed a seated position next to the immersion tank for the first hour of the experiment. Subsequently, they were immersed up to their necks, maintaining the same seated position in thermoneutral water ($35.0 \pm 0.2^\circ\text{C}$) for 1 hour followed by an additional hour sitting outside the tank. Throughout the experiment 250 ml/hr of tap water was given orally. All patients were on a regular hospital diet. Data are presented as means \pm S.E.M. Statistical analysis was performed by Student's t-test or the Wilcoxon paired-sample test. Experimental protocols were approved by the institutional committee on the ethics of human investigation.

Results

Hypertensive patients displayed a sixfold increase in plasma ANF as compared to normotensive controls (61.7 ± 13.2 vs. 9.6 ± 1.0 fmol/ml, $p < 0.01$). A subgroup of untreated patients with essential hypertension had comparably high levels. Patients with congestive heart failure displayed an 8-fold increase in plasma-ANF (81.5 ± 32.7 fmol/ml, 0.01). ANF plasma levels in cirrhotic patients (10.3 ± 1.3 fmol/ml) were not lower than in normotensive controls. ANF plasma levels in CHF patients were positively and significantly correlated to increased right atrial pressure and pulmonary capillary wedge pressure ($r = +0.72$, $p < 0.01$ and $r = +0.73$, $p < 0.01$) and inversely related to cardiac index ($r = -0.73$, $p < 0.01$). In the course of 6 months' therapy with an ACE inhibitor (enalapril), ANF plasma levels in CHF patients fell parallel to the hemodynamic improvement to 19% of pretreatment levels (from 81.5 ± 32.7 to 15.5 ± 4.2 fmol/ml, $p < 0.01$). Head-out water immersion induced an increase in ANF plasma levels by 83% following 1 hour of immersion (from 6.5 ± 0.8 to 12.0 ± 2.6 fmol/ml, $p < 0.01$) (Fig. 2). The increase in ANF plasma levels was accompanied by a marked renal response [15]. Cirrhotic patients without ascites displayed a similar increase in their ANF plasma levels under head-out water immersion, while cirrhotic patients with ascites showed a blunted response (a 50% increase as compared to a 98% increase in patients without ascites) [13]. An initial structural analysis of plasma ANF was performed by use of HPGPC in all plasma extracts. As previously reported [7], in normotensive individuals, ir-ANF consisted exclusively of authentic 3080-dalton ANF-28, the biologically active processing product of pro-ANF [18] (Fig. 2). Such a molecular analysis in cirrhotic patients yielded a similar pattern. However, in these patients, trace amounts of a higher molecular weight material of an estimated MW of 15000 daltons was also present. This higher molecular weight portion of ir-plasma ANF did not increase in parallel to the rise in authentic ANF-28 induced by head-out water immersion in cirrhotic patients (Fig. 3). Ir-ANF in hypertensive patients comprised a peak coeluting with synthetic ANF-28 as in normotensive subjects and cirrhotics; in addition, 15000-dalton ANF immunoreactivity and ANF-immunoreactivity eluting in the void volume (MW > 50000 daltons) were also present (Fig. 4). In some CHF patients elevated ANF plasma levels were even primarily composed of such higher molecular weight forms. While total ANF im-

munoreactivity in plasma was reduced under therapy with an angiotensin converting enzyme inhibitor, molecular weight analysis revealed that it was primarily the higher molecular weight forms that are decreased rather than authentic ANF-28 (Fig. 5).

Discussion

Sensitive radioimmunoassays allow for the measurement of ANF in plasma of healthy subjects [7, 19]. While qualitative changes under various manipulations and differences in plasma levels between various groups of patients seem to be consistently found by different research laboratories, actual measurements may vary considerably from laboratory to laboratory. Chromatographic analysis indicates that ANF may partly be bound to larger plasma proteins (the "void volume peak" in Fig. 5) which may be lost to varying degrees with different extraction procedures. On the other hand recovery efficiency may be overestimated as the synthetic standard added to the plasma sample may not bind to its carrier proteins under in vitro conditions. If tracer degradation by plasma constituents or interference by plasma proteins in the incubation mixture can be excluded, an un-extracted assay may more accurately quantitate total, i.e. bound and unbound, ANF immunoreactivity in plasma [20]. The "International Collaborative Study of the Proposed International Standard for Atrial Natriuretic Factor on Behalf of the American Heart Association/International Society of Hypertension/World Health Organisation" [21] will help to clarify the existing discrepancies.

From earlier studies reporting decreased ANF atrial tissue levels in the BIO 14.6 strain of Syrian hamsters, a model of hereditary cardiomyopathy, it had been speculated that a deficiency in ANF may cause venous congestion and edema formation in heart failure [22]. However, low tissue levels of a secreted substance may not necessarily reflect low plasma levels as the former may be the consequence of an enhanced secretory activity and, in fact, accompany increased plasma levels. In this study, patients with congestive heart failure displayed 8-fold higher plasma levels than healthy control subjects. ANF plasma levels were significantly correlated to right atrial and pulmonary capillary wedge pressures; they were inversely related to cardiac index. Congestive heart failure has, of late, been treated with angiotensin converting enzyme (ACE) inhibitors [23]. Interestingly, therapy with an ACE inhibitor effected a

reduction in total plasma ANF immunoreactivity parallel to a hemodynamic improvement. The high levels of ANF in CHF not undergoing treatment are probably a physiological response to combat this condition. ACE inhibitors presumably exert their ameliorative action upon congestive heart failure by a reduction in angiotensin II levels. These compounds are also associated with a reduction in circulating catecholamines, a decrease of vasopressin production and a reduction in aldosterone levels [24]. This may lead to the clinical improvement and secondarily to a reduction in ANF release. These data also provide evidence that ANF may not only be a biochemical marker of the failing heart but also of utility for the monitoring of its therapeutic management.

A mixed group of hypertensive patients displayed on the average a sixfold increase in their ANF plasma levels, actual values ranging from normal to excessively elevated. It may be speculated that measurements of ANF plasma levels in hypertensive patients may help to discriminate between patients with concomitant hypertensive heart disease and those whose hearts are not yet affected.

Despite intensive study, mechanisms of disturbed renal sodium handling in patients with cirrhosis are not, as yet, completely understood. It has been hypothesized that a failure to adequately elaborate the natriuretic hormone contributes to sodium retention in cirrhotics [25]. However, our data demonstrate that ANF plasma levels in cirrhotic patients are not lower than in healthy counterparts. We utilized Gauer's [14] water immersion model to investigate the stimulus-response-coupling in cirrhotics as compared to healthy volunteers. An immediate increase in

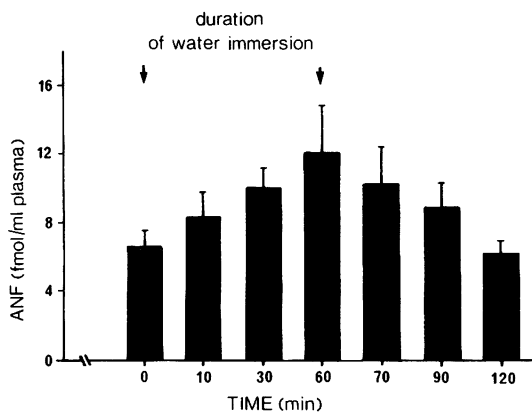


Fig. 1. ANF plasma levels before, during and after water immersion. Mean values and standard deviation of 12 healthy subjects

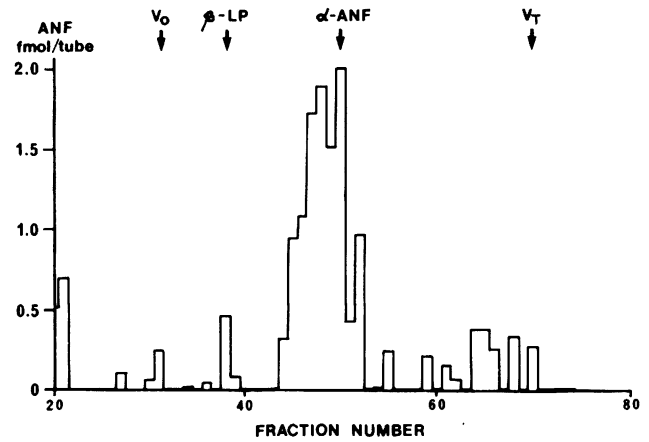


Fig. 2. Molecular weight pattern of ir-ANF in a representative healthy volunteer. The TSK-125 Bio Sil column was calibrated with BSA (V_0), leu-enkephalin (V_T), and a series of opioid peptides and synthetic ANF-28. Fraction aliquots were assayed for ANF immunoreactivity

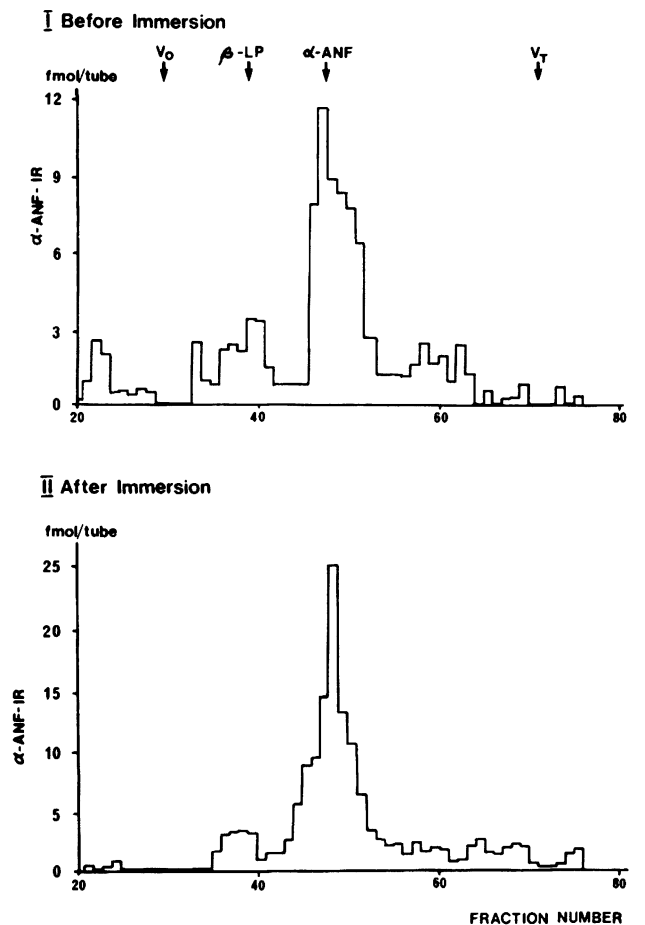


Fig. 3. Molecular weight pattern of ir-ANF in a patient with cirrhosis before (upper) and during volume augmentation by head-out water immersion (lower panel). For calibration details see text and Fig. 2. β -LP = β -lipotropin with a MW of 11 500 daltons was used for standardization

ANF plasma concentrations in normotensive controls (Fig. 1) was followed by a marked renal response [15]. There was no difference between normotensive controls and cirrhotic patients without ascites in their increase in ANF plasma levels under immersion. Conversely, patients with tense ascites tended to display a diminished increase in ANF [13]. This difference will be subsequently evaluated in more detail.

An initial structural analysis of circulating ANF was achieved by high performance gel permeation chromatography of plasma extracts. As previously described [7], ANF in healthy subjects eluted as a single 3080-dalton ANF peak parallel to synthetic ANF-28 (Fig. 2). Trace amounts of higher molecular weight activity of an estimated MW of 15 000 daltons (probably ANF-126) were detected in cirrhotic patients without ascites in ad-

dition to 3080-dalton ANF. Interestingly, head-out water immersion led to an increase in total ANF plasma immunoreactivity. Molecular weight analysis revealed that this increase in total ANF was exclusively caused by an increase in 3080-dalton ANF while 15 000-dalton ANF was essentially unchanged (Fig. 3). This finding is in favor of the contention that in these patients the stimulus-response-coupling is intact and the maximal processing capacity of the putative pro-ANF processing enzymes is not exceeded. Hypertensive patients (Fig. 4) and patients with congestive heart failure (Fig. 5) displayed a contrasting molecular weight pattern of their plasma ANF. In addition to authentic ANF-28, considerable amounts of ANF immunoreactivity eluted in the void volume (most likely ANF-28 bound to carrier proteins) and a third maximum eluted shortly before β -lipotropin (MW 11 500) at an estimated MW of 15 000 daltons. Further chromatographic analysis and, eventually, sequence analysis will reveal the precise structure of these higher molecular weight materials the biological role of which in plasma is at present unclear. The enalapril induced decrease in total plasma ANF, as may also be seen from Fig. 5, was primarily due to a reduction of 15 000-dalton ANF. This finding may indicate that the capacity of the putative processing enzymes may be exceeded in these patients resulting in the release of immature, unprocessed ANF. The hemodynamic improvement seen upon treatment with enalapril while not entirely reversing the symptomatology may lower the secretory stimulus for ANF release and thereby lead to a decreased release of unprocessed ANF. Further improvement, consequently, should eventually also lead to a decrease in the processing product.

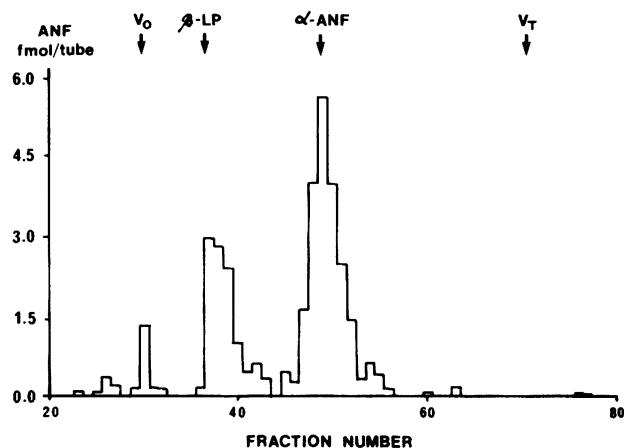


Fig. 4. Molecular weight pattern of ir-ANF in a hypertensive patient. For calibration details see figures above

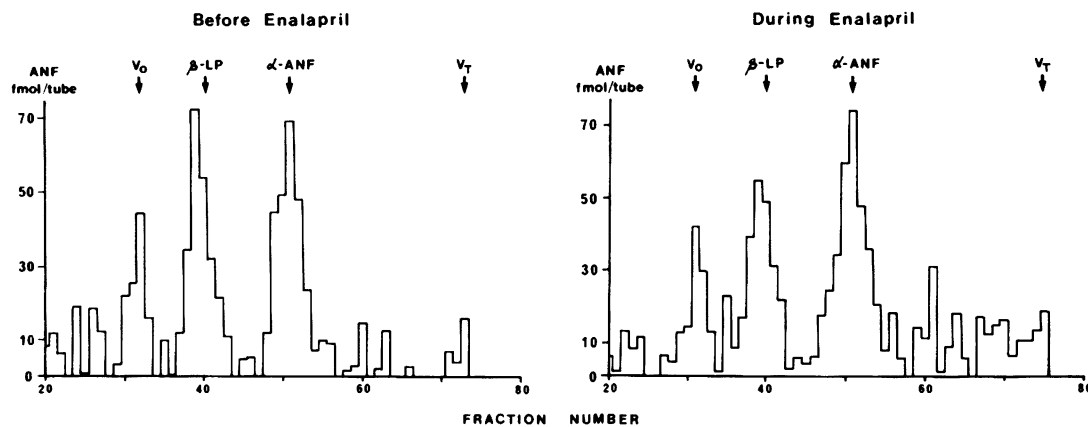


Fig. 5. Molecular weight pattern of ir-ANF in the plasma of a patient with CHF before (left) and two weeks following institution of therapy with enalapril (right panel). For calibration details see figures above

We conclude that a dysregulation of post-translational processing of pro-ANF may be an important feature in the pathophysiology of cardiovascular disease. These data also document that quantitation and characterization of ANF plasma levels will play an important role in the diagnosis of cardiovascular, renal and liver disease.

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Dr. Rainer M. Arendt
 Medizinische Klinik I
 Ludwig-Maximilians-Universität
 Klinikum Großhadern
 Marchioninstr. 15
 D-8000 München 70