Immunoreactive N-terminal pro-atrial natriuretic peptide in human plasma: plasma levels and comparisons with α-human atrial natriuretic peptide in normal subjects, patients with essential hypertension, cardiac transplant and chronic renal failure

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SUMMARY

1. Plasma levels of immunoreactive N-terminal pro-atrial natriuretic peptide (N-terminal ANP) have been measured in 25 normal subjects, 29 patients with essential hypertension, six cardiac transplant recipients, seven patients with dialysis-independent chronic renal failure and 11 patients with haemodialysis-dependent chronic renal failure. Plasma was extracted on Sep-Pak cartridges and N-terminal ANP immunoreactivity was measured using an antibody directed against pro-ANP (1-30).

2. Plasma levels of N-terminal ANP (means ± SEM) were 235.3 ± 19.2 pg/ml in normal subjects and were significantly raised in patients with essential hypertension (363.6 ± 36.3 pg/ml), in cardiac transplant recipients (1240.0 ± 196.2 pg/ml) in patients with chronic renal failure not requiring dialysis (1636.6 ± 488.4 pg/ml) and patients with chronic renal failure on maintenance haemodialysis (10336.1 ± 2043.7 pg/ml).

3. There were positive and significant correlations between the plasma levels of N-terminal ANP and α-human ANP (α-hANP) with individual correlation coefficients of 0.68 within the normal subjects, 0.47 in patients with essential hypertension, 0.78 in patients with chronic renal failure not requiring dialysis (1636.6 ± 488.4 pg/ml) and patients with chronic renal failure on maintenance haemodialysis (10336.1 ± 2043.7 pg/ml).

4. Gel filtration behaviour on Sephadex G-50 of the immunoreactive N-terminal ANP from Sep-Pak extracts of plasma from normal subjects or patients was consistent with a single peak having an elution volume corresponding to that of human pro-ANP (1-67) standard.

5. These studies demonstrate that the N-terminal pro-ANP peptide is co-secreted with α-hANP in both normal subjects and patients with cardiovascular/renal disease. The higher levels of the N-terminal ANP may reflect differences in the rate of elimination from the circulation but the exact structure and functional significance of the circulating N-terminal ANP remains to be established.

Key words: atrial natriuretic peptide precursor, cardiac transplant, cardiodilatin, essential hypertension, plasma, renal failure.

Abbreviations: ANP, atrial natriuretic peptide; α-hANP, α-human atrial natriuretic peptide.

INTRODUCTION

Since the discovery of the atrial natriuretic peptides (ANPs) (for review, see [1]), considerable work has been carried out to define their functional role in health and disease. Focus has centred primarily on the importance of the 28 amino acid peptide α-human ANP [α-hANP; ANP (99-126)] in cardiovascular and renal homoeostasis and in the control of sodium balance in particular (for review, see [2, 3]). α-hANP is stored within human atrial tissue as a high-molecular-weight precursor [pro-ANP (1-126)] derived from pre-pro ANP after cleavage of a signal peptide (see [4]), but little is known of the processing of the precursor or indeed of the functional significance of the N-terminal pro-ANP fragment. Although pro-ANP (1-126) is secreted from isolated cultured rat atrial cells (see [4]), there is little evidence to suggest that the precursor is activated within the peripheral circulation [5]. Nevertheless, it has been reported that N-terminal fragments [pro-ANP (1-30)] and
pro-ANP (31-67)] antagonized noradrenaline-induced vasoconstriction of isolated aortic tissue [6] and could also stimulate the activity of renal particulate guanylate cyclase [7], thereby raising the possibility of an important functional role. Knowledge of the existence and molecular forms of the N-terminal ANP in human plasma is therefore of interest, not only for a more detailed understanding of the secretion and processing mechanisms of α-hANP but also to explore further the physiological and pathophysiological importance of the N-terminal ANP. In the present study, therefore, we have investigated the existence and nature of the circulating N-terminal ANP in human plasma, not only in normal subjects but also in patients with essential hypertension, in cardiac transplant recipients and in patients with chronic renal failure.

METHODS

Subjects

Normal subjects. Twenty-five normal subjects (11 female, 14 male; 13 white, 12 black) with an average age of 38.4 years (range 18–61 years) were studied. All subjects were on their normal sodium intake and none was on drug treatment.

Essential hypertensive patients. Twenty-nine patients with established essential hypertension (12 female, 17 male; 23 white, six black) with a mean age of 51 years (range 27–70 years) were studied. Supine diastolic blood pressure measured by Arteriosonde under standardized conditions [8] was consistently greater than 90 mmHg (12 kPa). None of the patients had any evidence of cardiac or renal failure and none of the subjects was on active drug therapy.

Cardiac transplant recipients. Six male patients (five white, one black) with a mean age of 45.3 years (range 17–61 years) were investigated. These patients were on immunosuppressive therapy essentially as previously described [9] and at the time of study had no clinical evidence of heart failure or tissue rejection on recent biopsy.

Renal failure patients. Seven patients (three female, four male; five white, two black) with mean age of 61 years (range 40–73 years) had dialysis-independent chronic renal failure but were not on drug treatment at the time of the study. Glomerular filtration rate (ethylenediaminetetra-acetate method) ranged from 11.4 to 53.0 ml/min. Eleven other patients (seven female, four male; 10 white, one black) of mean age 56 years (range 36–74 years) with chronic renal failure were on regular haemodialysis. In these patients, plasma samples were collected before and after maintenance haemodialysis.

Blood and urine collection

Venous blood was collected into ethylenediaminetetra-acetate (potassium salt) tubes containing aprotinin (Trasylol; Bayer; 200 kallikrein inhibitory units/ml of blood) and centrifuged at 4°C (2000 g for 15 min). The plasma was removed and stored at −20°C for the measurement of ANPs. In some studies, 24 h urine collections were also made for the measurement of urinary sodium excretion.

Assay methods for plasma ANP

Peptide source and nomenclature. Synthetic peptide standards were obtained from Peninsula Laboratories Europe Ltd (St Helens, Merseyside, U.K.). The human ANP precursor has been referred to as pro-ANP (1–126) and other peptides are numbered according to this sequence numbering [10]. The C-terminal α-hANP is defined as ANP (99–126).

Extraction procedure. For the measurement of N-terminal ANP, plasma was extracted in duplicate (2.0 ml) on Sep-Pak C18 cartridges (Waters Associates) activated with 5 ml of methanol followed by 5 ml of distilled water. Plasma was drawn through the cartridge under vacuum. The cartridge was washed with 5 ml of distilled water, excess water was removed by passing air through the cartridge and the adsorbed material was eluted from the cartridge into glass tubes containing 100 μl of bovine serum albumin (radioimmunoassay grade, 1 mg/ml) with 4.5 ml of acidified ethanol (4% acetic acid/80% ethanol in water). All extracts were dried down under constant stream of air in a water bath at 50°C. The dried extracts were reconstituted in radioimmunoassay buffer A (100 mmol/l sodium phosphate, 50 mmol/l sodium chloride, 1 g of bovine serum albumin/l, 1.54 mmol/l sodium azide, 1 ml of Triton X-100/l, pH 7.3) in volumes of 250, 500, 750 or 1000 μl depending upon the source of plasma.

Radioimmunoassay. N-terminal ANP antibody [rabbit anti-pro ANP (1–30)] and iodinated pro-ANP (1–30) (1425 Ci/mmol) were obtained from Peninsula Laboratories Europe Ltd (St Helens, Merseyside, U.K.). This antibody displays 100% cross-reactivity with pro-ANP (1–30), 22% with pro-ANP (1–67) (cardiodilatin), but no cross-reactivity with C-terminal ANP (99–126). The iodinated N-terminal ANP was dissolved in 100 mmol/l sodium phosphate/50 mmol/l sodium chloride, pH 7.3 (buffer B) and stored frozen in small portions. Standard curves for the N-terminal ANP assay were constructed using synthetic pro-ANP (1–30) diluted in radioimmunoassay buffer A over a range of 78.1–5000 pg/tube (seven points). Incubations were set up (in duplicate) with 100 μl of standards or extracts, 100 μl of pro-ANP (1–30) antibody and 100 μl (approximately 6000 c.p.m.) of iodinated pro-ANP (1–30). Total binding was determined in the absence of standard (buffer alone) and non-specific binding was determined in the presence of excess standard (200 ng/tube). Standard curve and test samples were incubated overnight at 4°C and separation of free from bound antigen was carried out by precipitation with 50 μl of γ-globulin (10 mg/ml) and 800 μl of polyethylene glycol 6000 (22.5%, w/v) in buffer B. The tubes were vortex-mixed and centrifuged (2000 g for 15 min). The supernatants were aspirated and the pellets (bound fraction) were counted in a NE 1600 gamma counter. The limit of sensitivity of the assay was 78.1 pg/tube. Intra-assay and interassay coefficients of variation
were 12.0% \((n = 9)\) and 15.6% \((n = 10)\), respectively. The recovery of pro-ANP \((1-30)\) from human plasma through the extraction procedure, determined by the addition of various amounts \((0.71-11.76 \text{ nCi})\) of iodinated pro-ANP \((1-30)\) to human plasma, was independent of the load and averaged 58.3 ± 0.7% \((\text{mean ± SEM}, n = 15)\). Plasma values of immunoreactive N-terminal ANP are given in pg/ml and are not corrected for recovery.

Plasma levels of α-hANP were measured by radio-immunoassay after extraction on Sep-Pak cartridges as previously described [11].

**Gel filtration of extracted plasma.** Gel filtration was carried out on a column \((\text{Pharmacia})\) of 30 cm x 1.6 cm internal diameter at 4°C packed with fine Sephadex G-50 \((\text{particle size diameter 20-80 µm})\) in buffer B. The bed height of the column was 29 cm; elution was carried out under gravity at a flow rate of 6.25 ml h⁻¹ cm⁻¹ and 1 ml fractions were collected. Pooled plasma \((200 \text{ ml})\) from normal subjects was extracted on Sep-Pak C18 cartridges \((10 \text{ ml per cartridge})\) and treated as described above in the Extraction procedure section. All extracts were then reconstituted in a small aliquot of radioimmunoassay buffer A \((500 \mu l)\); this was achieved by transferring the aliquot from one tube to the next and vortex mixing. All tubes were rinsed with a further 500 µl portion of the same buffer; both extracts were then pooled and made up to a final volume of 1.0 ml with radioimmunoassay buffer A. Similar extracts were prepared using individual plasma from two patients with chronic renal failure \((5 \text{ ml of plasma})\) and from 20 ml of plasma from a cardiac transplant recipient. An aliquot \((0.5 \text{ ml})\) of each extract was used for gel filtration. The column was calibrated with pro-ANP \((1-30),\text{ ANP (99-126) and pro-ANP (1-67). Aliquots (0.5 ml) were used each containing 25, 5 and 500 ng for pro-ANP (1-30), for ANP (99-126) and pro-ANP (1-67), respectively. The void volume was estimated with 0.5 ml of Dextran Blue \((2 \text{ mg/ml}; \text{molecular weight } 2 \times 10^6)\). After gel filtration, the fractions collected were tested for both N-terminal and C-terminal immuno-reactivity using 100 µl volumes.

**Statistical analysis**

Statistical significance was assessed with paired or unpaired t-tests as appropriate, but non-parametric tests were also used in the presence of unequal group variances. A \(P\) value of less than 0.05 in both tests was taken to indicate a statistically significant comparison. Associations were tested with Spearman correlation coefficients. All statistical analysis was carried out on the Statistical Package for the Social Sciences [12] at the University of London Computer Centre. Means ± SEM are given as an indication of location and dispersion.

**RESULTS**

**Plasma levels of immunoreactive N-terminal and C-terminal ANP**

**Normotensive subjects.** The mean plasma level of N-terminal ANP in 25 normal subjects was 235.3 ± 19.2 pg/ml, approximately 20-fold higher than the corresponding value for C-terminal ANP (Table 1). In this group there was no significant difference in the plasma level of N-terminal ANP between male and female and between black and white subjects, but there was a significant correlation between the plasma level of N-terminal ANP and the age of the subjects \(\left(r = 0.40; \text{ } P < 0.05\right)\). All subjects were on their normal sodium intake and 24 h urinary sodium excretion, available in 20 subjects, was 145.0 ± 15.9 mmol.

**Essential hypertensive patients.** Plasma levels of both N-terminal and C-terminal ANP were significantly raised in the 29 patients with essential hypertension when compared with the levels in the normal group (Table 1). There was no significant difference in the ratio of N-terminal to the C-terminal ANP plasma levels when compared with the corresponding ratio in the normotensive group (Table 1). All subjects were on their normal sodium intake and 24 h urinary sodium excretion, available in 19 patients, was 143.4 ± 17.6 mmol.

**Cardiac transplant recipients.** Plasma levels of N-terminal and C-terminal ANP in the six cardiac transplant recipients were considerably elevated (fivefold) when compared with the values in the normal group (Table 1). The ratio of the N- to the C-terminal ANP plasma levels was slightly higher compared with that in the normotensive group, but this did not achieve statistical significance (Table 1).

**Dialysis-independent chronic renal failure patients.** The mean plasma level of immunoreactive N-terminal ANP in these patients was approximately sevenfold

<table>
<thead>
<tr>
<th>Plasma source</th>
<th>N-Terminal ANP (pg/ml)</th>
<th>C-Terminal ANP (pg/ml)</th>
<th>N-Terminal ANP/C-Terminal ANP ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects ((n = 25))</td>
<td>235.3 ± 19.2</td>
<td>12.5 ± 0.96</td>
<td>20.3 ± 1.9</td>
</tr>
<tr>
<td>Essential hypertensive patients ((n = 29))</td>
<td>363.6 ± 36.3**</td>
<td>23.9 ± 2.9**</td>
<td>17.8 ± 1.6</td>
</tr>
<tr>
<td>Cardiac transplant recipients ((n = 6))</td>
<td>1240.0 ± 196.2***</td>
<td>52.9 ± 13.0***</td>
<td>32.0 ± 8.1</td>
</tr>
<tr>
<td>Dialysis-independent chronic renal failure patients ((n = 7))</td>
<td>1636.6 ± 488.4***</td>
<td>63.6 ± 24.9***</td>
<td>36.3 ± 6.5***</td>
</tr>
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higher than that in the normotensive group (Table 1). Similarly, these patients also had raised levels of C-terminal ANP and a raised ratio of N-terminal to C-terminal ANP (Table 1).

**Dialysis-dependent chronic renal failure patients.** The patients with haemodialysis-dependent chronic renal failure had the highest values of N-terminal ANP. Average pre-dialysis values of N-terminal ANP ($10.336.1 \pm 2043.7$ pg/ml) were 44-fold higher than the levels in the normotensive subjects ($P<0.001$). These patients also had the highest values of C-terminal ANP ($350.7 \pm 77.9$ pg/ml; $P<0.001$ vs the normotensive group). After dialysis, there were no significant changes in the plasma levels of N-terminal ANP, but by contrast there was a significant reduction in the levels of C-terminal ANP (Fig. 1). When compared with the normal group, these patients also displayed a significantly ($P<0.01$) raised ratio of N-terminal to C-terminal ANP with values of $41.7 \pm 8.3$ before and $61.9 \pm 12.7$ after dialysis, respectively.

**Relationship between plasma N-terminal and C-terminal ANP**

There were significant correlations between plasma N-terminal and C-terminal ANP values for all subjects with an average correlation coefficient of 0.80 ($n=89$; $P<0.001$). Individual group correlation coefficients between the N- and C-terminal ANP values were 0.68 within the normal group and 0.47 within the hypertensive group (Fig. 2); similarly, there were significant correlation coefficients for the patients with dialysis-independent ($r=0.78$) and those with haemodialysis-dependent ($r=0.68$) chronic renal failure (Fig. 2; $P<0.05$ in every group), but the coefficient ($r=0.43$) for the cardiac transplant group, although positive, did not achieve statistical significance.

**Gel filtration of extracted plasma**

After gel filtration of the extracted plasma from normal subjects or from patients with cardiac transplant or renal failure, N-terminal ANP immunoreactivity occurred at an elution volume of 21–23 ml, corresponding to the elution volume of the synthetic human pro-ANP (1–67) standard (Fig. 3). No N-terminal ANP immunoreactivity was detected in those fractions corresponding to the elution

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**Fig. 1.** Plasma levels of N-terminal ANP (a) and C-terminal ANP (a-hANP; b) in 11 patients with dialysis-dependent chronic renal failure before and after maintenance haemodialysis. Results are shown as individual values and as means ± SEM. Abbreviation: NS, not significant.

**Fig. 2.** Plasma levels of C-terminal ANP (a-hANP) plotted against corresponding values of N-terminal immunoreactive ANP (a) in normotensive subjects (○) and patients with essential hypertension (●), and (b) in patients with dialysis-independent chronic renal failure (○) and patients with dialysis-dependent chronic renal failure before dialysis (●). See the text for further details.
DISCUSSION

In the present study we have developed a method for the measurement of the N-terminal fragment of the ANP precursor in human plasma. The method is based on plasma extraction on Sep-Pak cartridges and an antibody directed against pro-ANP (1-30). We have measured N-terminal ANP immunoreactivity in normal subjects, essential hypertensive patients, cardiac transplant recipients and in patients with renal disease. The major findings were the presence of a significant correlation between N- and C-terminal ANP levels and that the plasma levels of N-terminal ANP were considerably higher (about 20-fold in normal subjects) than the corresponding levels of α-hANP. An estimate of a more exact molar ratio, however, would depend on the form of the circulating immunoreactive N-terminal ANP and its cross-reactivity with the antibody used in the present study. Two recent studies [13, 14] have also measured plasma levels of N- and C-terminal ANP in normal subjects and also found considerably higher levels of N-terminal ANP. The absolute values reported for N-terminal ANP, however, were higher (greater than twofold) than those found in the present study. These discrepancies more than likely reflect differences in methods (i.e. extraction versus direct assays). The values given in the present study were not corrected for recovery and this may account, to some extent, for the lower values obtained. In addition, since the exact form of the circulating N-terminal ANP is still unknown, the use of different antibodies directed against different segments of the pro-ANP peptide may also be important. Gel filtration studies in the present study (Fig. 3) indicate a single peak eluting in parallel with the synthetic pro-ANP (1-67) (molecular weight 7368), suggesting a high-molecular-weight form. This is in good agreement with other recently published studies [13, 14]. By contrast, Winters et al. [15] suggested the presence of two N-terminal ANP fragments [pro-ANP (1-30) and pro-ANP (31-67)]. However, this was not confirmed by appropriate chromatography and the possibility that the same higher-molecular-weight form was actually being measured by the two different radioimmunoassay procedures used in their study cannot be excluded entirely.

The mechanisms and location of the conversion of the ANP precursor to N-terminal ANP and the active α-hANP is still unresolved. The atrial ANP precursor is stored within atrial granules (see [4]). Studies in the rat demonstrate minimal activation of the ANP precursor in whole blood [5]. The presence of a high-molecular-weight N-terminal segment in the circulation therefore suggests that the processing of the precursor occurs during the secretion process, perhaps at the plasma membrane level [16]. This is supported by the observation of equivalent plasma levels of the immunoreactive N- and C-terminal ANP in coronary sinus plasma [15]. Since the plasma levels represent the combined rates of secretion and elimination, the differences in the plasma levels may reflect differences in clearance rates. α-hANP is rapidly removed from the circulation with a short elimination volume of the shorter N-terminal [pro-ANP (1-30)] standard (Fig. 3). Measurement of C-terminal ANP (Fig. 3), also carried out on the same fractions, revealed a single peak of immunoreactivity corresponding to the elution volume of the standard ANP (99-126).
half-life (see [2, 3]). Limited information is available on the clearance of the N-terminal fragment, but work in the rat [5] suggests that it is eliminated from the circulation at a much slower rate, thereby accounting for the higher plasma levels. The kidney plays an important role in the elimination of the C-terminal ANP peptide (see [2, 3]), and given the relatively higher levels of N-terminal fragment. Plasma levels of C-terminal ANP were considerably elevated in patients with chronic renal failure. The extremely high levels in dialysis-dependent patients (Fig. 1) may be related to the volume overload, as after dialysis there was a significant reduction in plasma α-hANP. By contrast, there was no significant change in the circulating levels of N-terminal ANP (Fig. 1). Several explanations may account for this. Although the possibility of selective filtration of the lower-molecular-weight C-terminal ANP across the dialysis membrane cannot be excluded entirely, the absence of a significant reduction in the levels of the N-terminal ANP may also represent different clearance mechanisms for N- and C-terminal ANP peptides. Similarly, raised levels of both N- and C-terminal ANP were found in patients with chronic renal failure not requiring dialysis (Table 1). These raised levels may well be important in the renal adaptations to the reduced renal function. In fact, a recent study suggested that plasma ANP (α-hANP) was a major determinant of the control of renal sodium excretion in response to sodium loading in patients with renal failure [17].

Plasma levels of C-terminal ANP were also elevated in patients with cardiac transplants when compared with normal subjects (Table 1). Similarly, the levels of N-terminal ANP were also raised. The reasons for these raised levels are not clear but may reflect a number of possibilities including the increased amount of atrial tissue after cardiac transplantation, the presence of drug therapy or perhaps a loss of neuronal inhibitory influence on the release of ANP from the heart.

In previous studies [18] we have demonstrated raised levels of α-hANP in patients with established essential hypertension. In the present work (Table 1) we have also found raised levels of N-terminal ANP in these patients. As the ratio of the levels of N-terminal to C-terminal ANP were similar in magnitude to those in the normotensive group, this would be consistent with an increase in pro-ANP secretion possibly secondary to raised central venous pressures as discussed previously [18].

The simultaneous secretion of both N- and C-terminal ANP, in general, is consistent with the situation in other precursor-derived protein hormone systems [19, 20]. However, the structure and functional significance of the circulating N-terminal ANP remains to be determined. A recent study [7], however, reported that N-terminal pro-ANP fragments (1–30), (31–67) and (79–98) could activate particulate guanylate cyclase of isolated renal membranes from rat, rabbit and dog, raising the possibility of the kidney being a target organ for N-terminal ANP. But, as yet, there is no evidence for the presence of specific N-terminal ANP receptors within the kidney or within other potential target organs such as the adrenal and the vascular system. Nevertheless, the measurement of plasma N-terminal ANP provides a new tool for future studies on the physiological and pathophysiological importance of the ANPs.

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REFERENCES

N-terminal atrial natriuretic peptide in human plasma


