Circulating Immunoreactive proANP(1-30) and proANP(31-67) in Sedentary Subjects and Athletes

Elio F. De Palo,1* Wolfgang Woloszczuk,2 Martina Meneghetti,1 Carlo B. De Palo,1 Henning B. Nielsen,3 and Niels H. Secher3

Background: Atrial natriuretic peptide (ANP) is synthesized and stored in myocytes as prohormone (1-126), which upon release is cleaved into proANP(1-98) and α-ANP(99-126). In addition, cleavage of proANP(1-98) produces proANP(1-30), proANP(31-67), and proANP(79-98) fragments. ProANP(1-30) and proANP(31-67) have roles in fluid and electrolyte homeostasis. The aim of the present study was to develop a plasma assay for proANP(1-30) and proANP(31-67) and to compare results in trained athletes and sedentary subjects.

Methods: Two competitive enzyme immunoassays were established with affinity-purified sheep antiserum against synthetic ANP fragments. The immunoreactivity (ir) of proANP(1-30) and proANP(31-67) was measured in 10-μL plasma samples without extraction in a microwell-based assay. Plasma concentrations in sedentary male subjects (n = 22) and male endurance athletes (n = 14) were examined.

Results: In the assay for ir-proANP(1-30) and ir-proANP(31-67), the concentrations at 95% B/B₀ were 4.7 and 14.2 pmol/L, respectively. Within-run CVs were 4–6% and 5–6%, and between-run CVs were 9% for both assays. Both assays were linear on dilution (y = 0.9945x – 0.7291 and y = 1.0001x – 3.428), and the recoveries were 102–112% and 102–106%, respectively. In the sedentary and athletic groups, the ir-proANP(1-30) concentrations were similar: 318 ± 38 pmol/L and 312 ± 25 pmol/L (mean ± SE), respectively, whereas the ir-proANP(31-67) was higher in the rowers (713 ± 81 pmol/L) than in the sedentary subjects (387 ± 71 pmol/L; P < 0.005).

Conclusions: The proANP fragment assays are precise (CV <10%) and exhibit nearly quantitative recovery (102–112%). Only ir-proANP(31-67) responds to physical training.

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Atrial natriuretic peptide (ANP) 4 was first described by De Bold et al. (1). Since then, a family of natriuretic peptides has been characterized, and the structures of their active forms have been identified. In atrial myocytes, ANP is stored in secretory granules as a prohormone, proANP(1-126). When released, this peptide is cleaved into its active COOH terminus (α-ANP) and a second larger fragment, the N-terminal proANP(1-98), in equimolar amounts. The N-terminal fragment is further proteolytically cleaved in plasma to form the peptides proANP(1-30), proANP(31-67), and proANP(79-98) (2, 3). Winters et al. (4) suggested that both the NH₂ and COOH termini of the proANP are released simultaneously with exercise; in addition, Macaulay et al. (5) agreed that the peptides are cosecreted from the heart. However, the metabolism of these circulating fragments is unknown. The main stimulus for the secretion of natriuretic peptides is atrial stress in response to increased intracardiac volume (6–8), and their effects are natriuresis and vasodilatation (9).

The release of ANP increases in response to physical exercise (10, 11), reflecting increased venous return to the heart and perhaps the higher heart rate. Freund et al. (10) observed that, during marathon runs, the release of ANP increased in a dose- and time-dependent manner and returned to values nearer basal values by the end of the exercise.

Measurement of the N-terminal proANP requires an immunoradiometric assay. Numata et al. (12) established a two-step assay that uses monoclonal antibodies and synthetic N-terminal proANP(1-67). During physical ex-

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1 Section of Clinical Biochemistry, Department of Medical Diagnostic Sciences and Special Therapy, University of Padua, 35100 Padua, Italy.
2 Ludwig Boltzmann Institut für Experimentelle Endokrinologie, A-1100 Wien, Austria.
3 Department of Anesthesia, University of Copenhagen, DK-2100 Copenhagen, Denmark.
*Author for correspondence. Fax 039-49-657391; e-mail depalo@ux1.unipd.it.
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4 Nonstandard abbreviations: ANP, atrial natriuretic peptide; EIA, enzyme immunoassay; and ir, immunoreactive.
exercise, increases in the plasma α-ANP [proANP(99-126)] concentration also suggests that atrial proANP(1-126) synthesis increases, leading to increased formation of all fragments. The N-terminal proANP fragments have longer half-lives in the circulation than α-ANP, with corresponding higher plasma concentrations. Little is known about the metabolism of proANP(1-98), and the structures of the circulating forms are unclear. Nevertheless, the proteolytic cleavage could be in equimolar quantities, and the different circulating concentrations of the proANP fragments reflect their degradation and clearance rates (13). Furthermore, the responses of proANP peptides to various stimuli seem slow both when ANP increases or decreases (14, 15). The aim of this study was to develop enzymes immunoassays (EIAs) for plasma immunoreactive (ir)-proANP(1-30) and ir-proANP(31-67) without extraction. These new detailed EIAs were also devised to determine whether a difference in the circulating concentrations of these peptides exists between trained athletes and sedentary healthy subjects.

**Materials and Methods**

**PEPTIDES AND REAGENTS**

Human N-terminal proANP(1-30), middle proANP(31-67), C-terminal biotinylated proANP(1-30), and C-terminal biotinylated proANP(31-67) were purchased from Pichem. For cross-reactivity tests, proANP(79-98), α-ANP(99-126), and proANP(1-98) were purchased from the Institute of Mikrobiologie und Genetik der Universität Wien. A solution of biotinylated peptides with assay buffer was prepared before each assay. Peroxidase-streptavidin was purchased from Southern Biotechnology Associates. 3,3',5,5'-Tetramethylbenzidine solution was purchased from Sigma. The stopping solution was 3 mol/L sulfuric acid in distilled water.

**BUFFERS**

Buffer A (for coating) was 0.05 mol/L sodium borate buffer, pH 9.6. Buffer B (for washing) was sodium phosphate-buffered saline (0.06 mol/L sodium phosphate), pH 7.4, containing 0.1 mL/L Triton X-100. Buffer C (for blocking) was sodium phosphate-buffered saline (0.06 mol/L sodium phosphate), pH 7.5, containing 1 g/L nonfat dried milk (Skim Milk Powder; Fluka), 20 g/L fetal calf serum (JRH), 20 g/L peptone (Merck), and 20 g/L Karion F (Merck). Buffer D (assay buffer) was sodium phosphate-buffered saline (0.05 mol/L sodium phosphate), pH 7.4, containing 5 g/L bovine serum albumin.

**ANTIBODIES**

Donkey anti-sheep IgG Fc-specific antiserum was purchased from Guilday. The sheep antibody against proANP(1-30) IgG and the sheep antibody against proANP(31-67) IgG were purified by immunoaffinity chromatography on HiTrap minicolumns (Pharmacia) according to the protocol of the manufacturer. In brief, 0.5 mg of each peptide was bound to the column. The immunogen was column-coupled using 1 mL of the ligand solution (0.5 g/L immunogen in 0.2 mol/L sodium bicarbonate and 0.5 mol/L sodium chloride, pH 8.3). After the column was washed and non-coupled active groups were deactivated, 10 mL of the proANP-specific antiserum (diluted 1:2 in 50 mmol/L sodium borate buffer, pH 7.0) was loaded (0.5 mL/min) onto the column at room temperature. After suitable column washing, the proANP-specific antibody was eluted with sodium citrate buffer (100 mmol/L; pH 1.7). Fractions (0.5 mL) were collected in tubes containing 0.5 mL of 500 mmol/L sodium borate buffer (pH 10.0) for immediate neutralization. The protein concentrations of the eluted IgG-containing fractions were determined with a commercial protein assay (Pierce).

**MICROTITER PLATES**

Microwells (6.5 mm diameter; Nunc Maxisorp High Binding) were coated with 200 μL of diluted donkey anti-sheep IgG Fc-specific antiserum (200 ng/well) in buffer A overnight at 4 °C. After the wells were washed, they were blocked using buffer C (350 μL). The microwells were then washed with buffer B (350 μL), dried, and stored at 4 °C before use. Before the assay, the microwells were coated with the appropriate proANP antibody (200 μL; 0.5 ng/well in buffer D) by incubation for 5 h at room temperature.

**SUBJECTS AND PLASMA SAMPLE PREPARATION**

Blood samples were drawn from an antecubital vein after the subjects had been supine for at least 15 min. Vacutainer Tubes containing heparin were used. The blood was placed on ice and centrifuged (800–1000g) for 15–20 min; the plasma samples were stored at −80 °C.

Blood samples were obtained from internationally competitive male rowers (14 males) and from age-matched sedentary control subjects (22 males). Table 1 shows the mean (± SE) ages, weights, height, blood pressure, and heart rates of the athletes and control subjects.

<table>
<thead>
<tr>
<th>Group studied</th>
<th>Age, years</th>
<th>Height, cm</th>
<th>Weight, kg</th>
<th>Blood pressure, mmHg</th>
<th>Heart rate, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Athletes</td>
<td>25 ± 1</td>
<td>182 ± 2</td>
<td>74.4 ± 0.5</td>
<td>Systolic 127 ± 7</td>
<td>52.6 ± 0.6</td>
</tr>
<tr>
<td>Controls</td>
<td>24 ± 1</td>
<td>179 ± 2</td>
<td>79.9 ± 2.9</td>
<td>Systolic 132 ± 3</td>
<td>Diastolic 64 ± 1</td>
</tr>
</tbody>
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*P < 0.005.
All subjects fasted overnight, and the blood samples were collected in the morning (0900–1100). A repeat blood collection was obtained from seven randomly chosen athletes on a second day under the same conditions. The Ethics Committee of Copenhagen approved the experimental procedures.

EIA assays
Calibrators for proANP(1-30) (range, 0–600 pmol/L) and proANP(31-67) (range, 0–1250 pmol/L) were prepared in assay buffer. The coated wells were incubated with the sheep antibody directed against proANP for 5 h at room temperature as described. These antibody solutions were then discarded, the wells were washed, and the proANP calibrators, diluted samples (1:20 in assay buffer), or controls (200 µL) were added to each well. Tracer solution [50 µL of 360 ng/L biotinylated proANP(1-30) and 3.6 µg/L biotinylated proANP(31-67)] was pipetted into the mixture and incubated overnight at room temperature. On the following day, the contents of the wells were discarded. After the wells were washed three times (350 µL each time), the peroxidase-conjugated streptavidin (200 µL) was added and incubated for 1 h with shaking at 37 °C. After the wells were washed four times (350 µL each time), 200 µL of 3,3',5,5'-tetramethylbenzidine solution was added. The reaction was stopped after 20–30 min by the addition of 50 µL of the sulfuric acid solution. The absorbance was read at 450 nm. Calibration curves were prepared using a logit/log curve (absorbance vs concentration).

Statistical analysis
The equation for the interpolation of the dose–response curve was computed with a four-parameter logistic function. Concentrations were calculated with commercial software. Detection limits were measured as the mean values at the 95% confidence limit of the absorbance of the St0 samples. ANOVA was used for the statistical analysis. \( P \leq 0.05 \) was considered statistically significant.

Results
Analytical evaluation
Calibration curves. For each proANP peptide, a calibration curve was prepared with synthetic peptides (Figs. 1 and 2). The concentration of peptide at 95% B/B0 was 2.4 pmol/L for proANP(1-30) and 10 pmol/L for proANP(31-67).

Precision. The CVs for the proANP(31-67) assay, assessed in 10 intrassay replicates of two peptide concentrations (545 and 1843 pmol/L) and in 6 different assays at 1885 pmol/L proANP(1-30), were 6%, 4%, and 9%, respectively. For 12 replicates of two proANP(31-67) concentrations (900 and 3400 pmol/L) and for six different assays at a peptide concentration of 3350 pmol/L, the CVs were 6%, 5%, and 9%, respectively.

Detection limit. The detection limits of the proANP(1-30) and proANP(31-67) assays were 4.7 ± 0.8 pmol/L (\( n = 8 \)) and 14.2 ± 2.1 pmol/L (\( n = 8 \)), respectively.

Dilution and recovery tests. Dilution curves of plasma samples gave linearity in the EIA measurements of proANP(1-30) and proANP(31-67). The equations for the two curves were: \( y = 0.9945x - 0.7291 \) and \( y = 1.0001x - 3.428 \) (Fig. 3). The recoveries of added proANP(1-30) and proANP(31-67) at different concentrations were 102–112% for 30 and 150 pmol/L proANP(1-30) and 106–102% for 25 and 750 pmol/L proANP(31-67).

Interference and cross-reactivity. Cross-reactivities between the two peptides and with proANP(79-98) and \( \alpha \)-ANP(99-126) were <1%. The two EIAs, Nt-ANP and md-ANP, recognized proANP(1-98) with 68% and 108% cross-reactivity, respectively.
concentrations in plasma samples

In the control subjects (n = 22), the ir-proANP(1-30) plasma concentration was 318 ± 38 pmol/L, whereas in athletes (n = 14), the concentration was 312 ± 25 pmol/L; there was no significant difference between these two groups. For ir-proANP(31-67), the plasma concentration was lower (P < 0.005) in the control group (387 ± 71 pmol/L) than in the athletes (713 ± 81 pmol/L).

The relationships between the concentrations of the two peptides in plasma obtained from control volunteers and athletes were assessed by the calculation of a correlation coefficient by linear regression (Fig. 4).

Discussion

This study demonstrated that the two EIA s had good performance for measurement of ir-proANP(1-30) and ir-proANP(31-67) in human plasma samples without prior extraction. Both assays can overcome the analytical problems involved with the determination of ANP(99-126) related to its short half-life. The cross-reactivity results indicate that the antibodies used react specifically with their epitopes. In fact, the anti-proANP(1-30) antiserum did not demonstrate cross-reactivity with α-ANP(99-126) hormone. As expected, proANP(1-98) demonstrated a cross-reactivity with the antiserum anti-proANP(31-67) and with the antiserum anti-proANP(1-30).

CONCENTRATIONS IN PLASMA SAMPLES

In the control subjects (n = 22), the ir-proANP(1-30) plasma concentration was 318 ± 38 pmol/L, whereas in athletes (n = 14), the concentration was 312 ± 25 pmol/L; there was no significant difference between these two groups. For ir-proANP(31-67), the plasma concentration was lower (P < 0.005) in the control group (387 ± 71 pmol/L) than in the athletes (713 ± 81 pmol/L).

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With regard to what these assays were actually measuring, it should be taken into account that the two utilized antibodies were immunoaffinity purified; the measured immunoreactivities recognized epitopes in the proANP(1-30) and proANP(31-67) peptides, but other circulating fragments, particularly proANP(1-98), were also measured. Furthermore, other peptides such as proANP(1-67) and proANP(31-98) might be carefully investigated.

The plasma proANP fragment immunoreactivity determination by specific antibodies suggests that the ir-proANP(1-30) is present at a lower plasma concentration than the ir-proANP(31-67). These results are in agreement with the findings of Numata et al. (12) and Winters et al. (3). In fact, the molar ratio of ir-proANP(31-67) to ir-proANP(1-30) was 2.3 ± 0.2 in the athletes and 1.2 ± 0.2 in the sedentary subjects. Thus, the ratio for the athletes was higher (P < 0.001) than that of the sedentary subjects. In any case, the good positive correlation (Fig. 4) between plasma concentrations of ir-proANP(1-30) and ir-proANP(31-67) and their ratio may suggest cosecretion but different half-lives of the fragments in the circulation, and renal clearance and protease activity might be involved (3). ProANP(31-67) has been shown to have a role in fluid and electrolyte homeostasis (13). Synthetic proANP(31-67) and α-ANP(99-126) are diuretic and natriuretic (14, 15). Thus, in agreement with the hypothesis of Winters et al. (3), it could be suggested that the same ANF prohormone, similarly to pro-opiomelanocortin, contains several hormones within its amino acid sequence.
Our findings demonstrate that the ir-proANP(31-67) plasma concentration is higher in trained athletes than in sedentary control subjects. This proANP fragment might change more slowly than α-ANP (16, 17). The ir-proANP(31-67) might be maintained at a high concentration as a consequence of the continuous daily stimuli of endurance training, but blood pressure might also be involved. In fact, a strong positive correlation with systolic and diastolic blood pressure has been observed in exercising healthy individuals (18). Stimulation of atrial ANP(1-126) synthesis and the release of α-ANP(99-126) and proANP-related fragments could be caused by atrial stretch and distension associated with physical activity. Proveda et al. (19) recently demonstrated that diastolic blood pressure at rest was lower in a group of trained than in control subjects. This finding is in agreement with the present data (P < 0.005; Table 1), which were generated not to study this application, but to suggest one of the uses of these analytical methods.

Among elderly women, differences in the response of the NH2-terminus of proANP were found that are suggested to reflect differences in postsecretory mechanisms (20). Other factors may include renal function and other hormones, as well as other biochemical variables of hemodynamic involvement.

In conclusion, this study reinforces the idea that proANP fragments with a longer half-life, such as proANP(1-30) and proANP(31-67), can be useful biochemical indices in a study of physical exercise. The measurements carried out on plasma samples obtained from healthy sedentary subjects and from athletes demonstrated no statistical difference for the ir-proANP(1-30), but plasma ir-proANP(31-67) was higher in the trained athletes than in the sedentary subjects. This finding suggests a use of these assays for evaluation of fluid homeostasis with physical exercise.

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References