Increased Release of the N-Terminal and C-Terminal Portions of the Prohormone of Atrial Natriuretic Factor During Immersion-Induced Central Hypervolemia in Normal Humans[1] (42990)

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Abstract. **The role of peptides from the N terminus and C terminus of the 126 amino acid atrial natriuretic factor (ANF) prohormone in modulating renal sodium and water handling has not been defined. Since water immersion to the neck (NI) provides an acute central volume expansion identical to that produced by 2 liters of saline but without plasma compositional change, immersion to the neck was used to assess the N-terminal and C-terminal portions of the ANF prohormone response to acute central blood volume expansion in seven seated sodium-replete normal subjects. Both the C terminus, which contains amino acids 99–126 and is identical to ANF, and the whole N terminus (i.e., amino acids 1–98) increased promptly with NI and peaked after 1 hr of immersion. A *M*, 3900 peptide from the midportion of the N terminus consistent with amino acids 31–67 (i.e., pro-ANF-31–67) also increased with NI and followed a pattern of increasing circulating concentration nearly identical to that of the whole N terminus of the prohormone, except that its maximal concentration was at the second hour of the 3 hr of NI. With cessation of immersion, ANF decreased to preimmersion levels within 1 hr whereas the N terminus and pro-ANF-31–67, although their circulating concentrations were decreasing, were still significantly elevated at 1 hr. These findings suggest that the increase in plasma ANF, the N terminus of the ANF prohormone, and pro-ANF-31–67 from the midportion of the N terminus, with natriuretic properties similar to ANF, contribute to the natriuretic response to NI, implying a physiologic role for these atrial peptides in modulating volume homeostasis in humans.** [P.S.E.B.M. 1989, Vol 192]

Atrial natriuretic factor (ANF), the 28-amino acid C-terminal end of the 126-amino acid ANF prohormone synthesized in cardiac myocytes, has potent natriuretic, vasodilatory, and renal hemodynamic effects (1–3). Recent evidence indicates that peptides from the N-terminal end of the prohormone, i.e., amino acids, 1–30 (pro-ANF-1–30) and amino acids 31–67 (pro-ANF-31–67) also vasodilate aortas *in vitro* (4) and that pro-ANF-31–67 has potent natriuretic, diuretic, and blood pressure lowering properties *in vivo* similar to those of ANF (5). The N terminus of the prohormone as well as the C terminus (i.e., amino acids 99–126) have recently been demonstrated to circulate normally in humans (6–10), dogs (11), and rats (12, 13). Although the physiologic regulation of ANF release in healthy subjects has been the subject of intense investigation (1, 2, 14–18) only rapid heart rates precisely increased via ventricular cardiac pacing is at present known to be a physiologic cause of release of the N terminus of the ANF prohormone (11).

Previous studies have demonstrated that head-out water immersion produces a prompt, marked, and sus-

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tained central hypervolemia without the necessity of infusing exogenous volume expanders and thus altering plasma composition (19–22). We and others have demonstrated that head-out water immersion produces a prompt release of atrial natriuretic factor that is associated with marked natriuresis (15–18, 23). This investigation was designed to determine whether the whole M_r 10,000 N terminus (i.e., amino acids, 1–98) of the ANF prohormone and/or a M_r 3900 peptide from the midportion of the N terminus consistent with pro-ANF-31–67 in addition to ANF (i.e., C terminus) are released with central hypervolemia induced by head-out water immersion.

Methods

Subjects. Seven male volunteers between the ages of 20 and 35 years (average, 28 ± 4 years) were investigated during head-out water immersion. For time controls an additional seven healthy male subjects (mean age, 27 ± 5 years) sat quietly outside the immersion tank for the 5-hr period of investigation. All had a negative history for hypertension, cardiovascular disease, and diabetes. Clinically, apparent renal disease was excluded in all subjects by documenting the presence of a normal urine sediment and creatinine clearance and the absence of proteinuria. The use of alcohol, tobacco, tea, coffee, and all medications was prohibited for at least 24 hr before and during each of the studies. The experimental protocols were similar and were carried out as follows.

Following 10 hr of overnight fluid restriction, each subject was instructed to sit quietly. At 0800 hr, after voiding and completely emptying his bladder, the subject once again assumed the seated position. Immediately after the subjects had voided, an 18-gauge, 1.5-inch Teflon catheter with a flash chamber and an accompanying Teflon stylet (Jelco, Raritan, NJ) was inserted into a forearm vein, permitting sequential sampling of blood without the use of an anticoagulant or an iv infusion. Venous blood was drawn using a prechilled 10-ml polystyrene disposable syringe. After each sample was obtained, a sterile stylet was inserted into the catheter. After a 60-min prestudy period, the immersion subjects sat quietly in the study tank immersed in water to the neck for 3 hr (0900–1200 hr); this was followed by a 1-hr recovery period of quiet sitting outside the tank. In order to establish an appropriate time control, an additional seven healthy male subjects were studied during an identical 5-hr period while seated in a chair outside the immersion tank. Blood samples were obtained before and after the study for sodium, potassium, osmolality, and creatinine determinations. Samples for immunoreactive ANF, immunoreactive pro-ANF-1–98, and immunoreactive pro-ANF-31–67 were obtained every 30 min during the prestudy period, at 15, 30, 60, 120, and 180 min of immersion, and at 15, 30, and 60 min during the recovery period. These blood samples for radioimmunoassay were drawn into chilled vacutainer tubes containing potassium EDTA. The plasma was then separated with a refrigerated centrifuge and flash-frozen with dry ice and acetone and stored at −76°C until radioimmunoassay evaluation. Each subject was requested to void spontaneously at hourly intervals during the studies. To void during the immersion period, the subjects stood briefly on a platform in the immersion tank after collection of blood. To maintain adequate urine flow, an initial waterload of 400 ml was administered, and subsequently 200 ml of water were ingested hourly. Sodium, potassium, osmolality, and creatinine were measured in all urine samples. Immersion was carried out in a waterproof tank described in detail previously (19–22). A constant water temperature of 34.5 ± 0.5°C was maintained by two heat exchangers, as detailed previously (19–22).

Radioimmunoassays to measure the N terminus of the prohormone were devised to amino acids 1–30 and 31–67 of the 126-amino acid prohormone while the C-terminal assay measures amino acids 99–126 of the prohormone, i.e., ANF, as described previously by our laboratory (6,7,11). Our pro-ANF-1–30 radioimmunoassay recognizes a component in plasma of approximately M_r 10,000 as characterized by G-50 Sephadex gel-permeation chromatography which is consistent with the whole N terminus (i.e., amino acids 1–98), but without the C terminus attached to it (24). The pro-ANF-31–67 radioimmunoassay recognizes in plasma a component of the N terminus of approximately M_r 3900 which is consistent with measuring only pro-ANF-31–67 (M_r 3878) (24).

For the radioimmunoassay of ANF, pro-ANF-1–98, and pro-ANF-31–67, the extracted plasma was first reconstituted in 100 μl of 0.1 *M* phosphate buffer (pH 7.4), containing 0.5 *M* NaCl, 0.1% bovine serum albumin, 0.1% Triton X-100, and 0.01% NaN_3. To the redissolved sample, 100 μl (0.03 mg) of rabbit IgG plus 100 μl of the respective antisera were added and incubated for 24 hr. Then 100 μl of the ^{125}I-labeled peptides (10,000 cpm) were added and incubated for 18 hr at 4°C. The precipitation of the antibody-bound tracer was accomplished by adding 100 μl of goat anti-rabbit globulin after the above described 18-hr period and incubating this mixture for 2 hr at room temperature. Each tube was then centrifuged at 3000*g* for 20 min. The supernatant was aspirated and the pellet counted in a gamma counter. All determinations were performed in triplicate. The intraassay coefficient of variation for pro-ANF-1–30, -31–67, and ANF radioimmunoassays were 4.8, 5.3, and 5.7%, respectively. The interassay coefficient of variation was 8% for both pro-ANF-1–30 and -31–67 radioimmunoassays whereas ANF radioimmunoassay's interassay variation was 6.9%.

Recovery was examined by adding synthetic unla-

beled pro-ANF-1–30, pro-ANF-31–67 and ANF at 100, 200, and 400 pg/ml to pooled human plasma. Recovery for pro-ANF-1–30 was 83.5 ± 13.2% (SD) and pro-ANF-31–67 recovery was 100.9 ± 8.9%. Recovery of ANF was 92 ± 11% (SD). The respective IC_{50}s were 180, 120, and 11 fmol/tube while the lowest detectable concentrations were 40, 35, and 1.4 fmol for pro-ANF-1–30, -31–67, and ANF, respectively. Serial dilution of pooled plasma has revealed excellent parallelism of standard and unknown in these assays (7, 11). Reverse phase high-pressure liquid chromatography utilizing Novapak C-18 (5 g) cartridge columns revealed that the pro-ANFs-1–30, -31–67, and -99–126 (ANF) measured were authentic.

In the presentation of the data, mean values are followed by the standard error of the mean as an index of dispersion. Data were evaluated statistically by analysis of variance (ANOVA) and by paired or unpaired *t* tests. Differences with $P < 0.05$ were considered significant.

Permission for the study was obtained from each subject after a detailed description of the procedure and potential complications. The protocol was approved by the Human Experimentation Committees of the University of Miami School of Medicine and the Miami Veterans Administration Medical Center and was in compliance with the principles set forth in the Declaration of Helsinki. No complications occurred.

Results

Plasma N-Terminal and C-Terminal ANF Prohormone Levels. Water immersion markedly stimulated the release of both atrial natriuretic factor (i.e., amino acids 99–126 of the prohormone) which is the C-terminal end of the prohormone and the N terminus of the prohormone which was evaluated by two separate radioimmunoassays which recognize the whole N terminus (i.e., amino acids 1–98) with the pro-ANF-1–30 radioimmunoassay and a small midportion of the N terminus consistent with amino acids 31–67 via the pro-ANF-31–67 radioimmunoassay (Fig. 1). As depicted in Figure 1, the circulating concentration of ANF and the N terminus of the prohormone as evaluated by pro-ANF-1–30 and pro-ANF-31–67 radioimmunoassays were remarkably constant in these seven individuals when sampled at 30-min intervals during the preimmersion hour, with the mean value for ir pro-ANF-1–98, -31–67, and -99–126 (ANF) being 478 ± 18 fmol/ml, 345 ± 42 fmol/ml, and 18 ± 1 fmol/ml, respectively. In response to immersion, the circulating concentration of the N terminus and C terminus of the prohormone began to increase soon after immersion with the first time period illustrated in Figure 1 being 15 min after immersion was begun. The mean circulating concentration of ANF was maximal at 1 hr of immersion (29 ± 2 fmol/ml), with its circulating concentration being similar at 2 hr of immersion (28 ± 3 fmol/l).

In contrast to the whole N terminus, the midportion of the N terminus of the prohormone did not peak until the second hour of immersion (Fig. 1). Thus, its circulating concentration when evaluated by the pro-ANF-31–67 radioimmunoassay had increased from 345 ± 42 fmol/ml (preimmersion) to 577 ± 54 fmol/ml at 1 hr and to 659 ± 41 fmol/ml at 2 hr of immersion ($P < 0.05$ for second hour versus first hour of immersion when evaluated by ANOVA). Pro-ANF-1–98 increased from its circulating preimmersion concentration of 478 ± 18 fmol/ml to 830 ± 53 fmol/ml after 1 hr of immersion and was 887 ± 51 fmol/ml after 2 hr of immersion ($P < 0.05$ and $P < 0.05$, respectively, versus preimmersion value). Although the circulating concentration of the whole N terminus (i.e., amino acids 1–98) was higher at 2 hr versus 1 hr of immersion (Fig. 1), this difference was not significant when evaluated by ANOVA.

Water immersion, thus, induced release of both the N terminus and the C terminus of the prohormone but the maximal increase in the circulating concentration was greater for the N terminus with pro-ANF-1–98 and -31–67 increasing 86 ± 8 and 101 ± 18% (SEM) versus 52 ± 12% (SEM) for the C terminus, i.e., ANF ($P < 0.005$). The augmentation of N terminus and ANF was sustained throughout immersion with the levels in all three radioimmunoassays remaining >50% above baseline at 180 min in all subjects. The renal response to immersion in these subjects has been previously published (17). Immersion induced a greater than 3-fold increase in urine flow rate (V) and a 2-fold increase in sodium excretion ($U_{na}V$), which peaked during the third hour of immersion (17).

In order to establish an appropriate time control, an additional seven healthy male subjects were studied during an identical 5-hr period while seated in a chair outside the immersion tank. The mean circulating concentration of the N terminus and C terminus of the ANF prohormone during this 5-hr period without immersion did not vary significantly as observed in Table I.

Cessation of water immersion resulted in a prompt return of the C terminus to prestudy levels (Fig. 1). The N terminus as evidenced by the pro-ANF-1–98 and pro-ANF-31–67 ir circulating concentrations, although decreasing immediately after stopping the 3 hr of immersion, remained elevated at 1-hr postimmersion versus their preimmersion concentrations (Fig. 1).

Discussion

The possible participation of cardiac atria in the control of extracellular fluid volume has been known for many years (21, 25). It is well recognized that mammalian atria have mechanoreceptors that are sen-

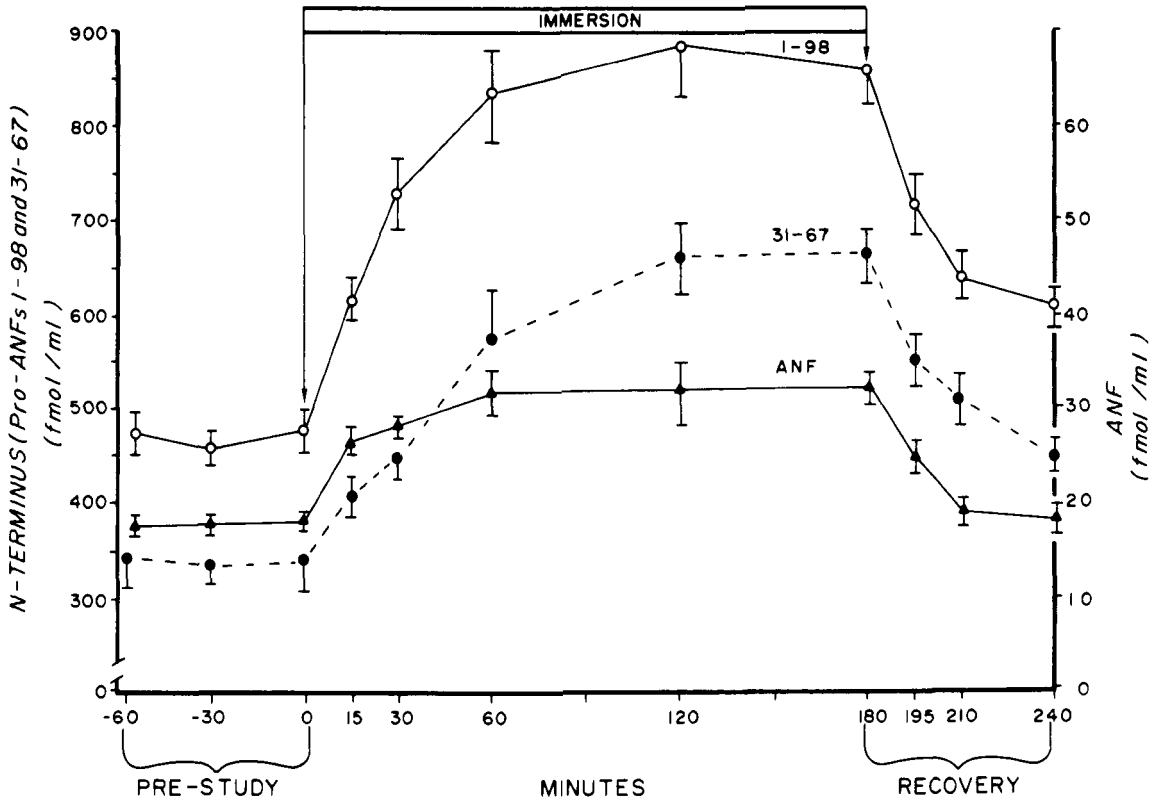

Figure 1. Water immersion to the neck releases both the N terminus and C terminus of the ANF prohormone. Within 15 min there was an increase in the circulating concentration of the whole 98-amino acid N terminus ( ), the C terminus (ANF,), and pro-ANF-31–67 ( ) of the ANF prohormone that was sustained throughout immersion. Postimmersion was associated with prompt return of ANF to the prestudy level but the whole N terminus and pro-ANF-31–67 were still elevated after 1 hr. Results are the mean ± SEM $*P < 0.05$ or more for the N and C terminus, and pro-ANF-31–67 from the midportion of the N terminus of ANF prohormone at the end of the study compared with the prestudy period when evaluated by the ANOVA.

Table I. Mean Circulating Concentration of the Whole N Terminus (i.e., Pro-ANF-1–98), Midportion of the N Terminus (i.e., Pro-ANF-31–67), and C Terminus (i.e., ANF) in Seven Seated Healthy Nonimmersed Volunteers Evaluated at the Same Time Periods as the Immersed Volunteers

Time (min)	Pro-ANF-1–98 (fmol/ml)	Pro-ANF-31–67 (fmol/ml)	ANF (fmol/ml)
−60	473 ± 21[a]	339 ± 31[a]	18 ± 1[a]
−30	460 ± 18	346 ± 43	17 ± 2
0	465 ± 27	342 ± 36	19 ± 2
15	459 ± 19	345 ± 40	18 ± 2
30	470 ± 16	337 ± 37	17 ± 1
60	474 ± 23	352 ± 33	19 ± 2
120	469 ± 22	356 ± 42	16 ± 2
180	458 ± 17	349 ± 45	17 ± 1
195	466 ± 24	361 ± 38	18 ± 3
210	475 ± 15	353 ± 41	17 ± 3
240	479 ± 26	359 ± 44	19 ± 2

[a] The values obtained over the 5-hr study period did not vary significantly when evaluated by ANOVA.

sitive to stretch or distensibility induced by volume changes (25, 26). The present studies demonstrate that central hypervolemia induced by head-out water immersion provokes a prompt and sustained increase in both the N terminus and the C terminus of the 126-amino acid ANF prohormone in normal human subjects. Head-out water immersion also was found to cause an increase in the circulating concentration of the midportion of the N terminus of the ANF prohormone that essentially parallels the circulating concentration of the whole N terminus. In view of the compelling evidence that the effects of water immersion are mediated by increases in central blood volume (20, 21), the present findings suggest that a volume-mediated increase in atrial stretch is one mechanism subserving the release of both the N terminus and the C terminus of the ANF prohormone. Whether pro-ANF-31–67 consisting of amino acids 31–67 of the N terminus is released simultaneously with the whole N terminus from the heart or if the N terminus is cleaved to form pro-ANF-31–67 in the circulation could not be determined from the present investigation. The present investigation does suggest, however, that if the N terminus is proteolytically processed in the circulation, that this processing occurs fairly soon after release since even at the early time points (15 and 30 min) after immersion began the circulating concentration of pro-ANF-31–67 was increased proportional to the increase of the whole N terminus. In a previous study of release of the N

terminus and C terminus secondary to cardiac pacing (11), we have found that at heart rates of 125 beats/min and above pro-ANF-31–67 as well as the whole N terminus are increased within 1 min of beginning pacing. Thus, if the whole N terminus is being proteolytically processed to form pro-ANF-31–67 this occurs within 1 min after being released from the heart.

The current observations are consistent with the postulate that increased plasma levels of peptides from the N terminus and C terminus of the ANF prohormone contribute to the natriuresis induced by immersion. Previous studies have demonstrated that the infusion of exogenous C terminus of the prohormone, i.e., ANF promotes a natriuresis in both humans (14) and experimental animals (1). The present study confirms earlier studies that plasma ANF (i.e., C terminus) increases with head-out water immersion (15–17, 23) but, in addition, demonstrates that the N terminus of the ANF prohormone is released with central hypervolemia. Since the N terminus contains at least one peptide, i.e., amino acids 31–67, that is natriuretic and diuretic (5) but which circulates normally 10 to 20 times longer than ANF (5, 11), it would appear that this peptide from the N terminus may also be important in acute volume homeostasis. With infusion of pro-ANF-31–67 into rats, its natriuretic and diuretic effects begin as early as ANF's (5). Since the peak concentration of pro-ANF-31–67 in the present investigations was at the same time as the peak natriuresis and diuresis (17), it may reflect the contribution of pro-ANF-31–67 to the diuresis and natriuresis.

In this investigation one can observe that the midportion of the N terminus peaked later than the C terminus during acute volume expansion induced by head-out water immersion. One might wonder if both ends of the prohormone are being released simultaneously why does the midportion of the N terminus peak later. The answer to this, we believe, may be due to its longer circulating half-life, with the midportion of the N terminus of ANF prohormone peak at the second hour of immersion versus 1 hr of immersion for the C terminus reflecting its slower clearance from the circulation. The site of clearance may also be important. The midportion of the N terminus appears to be cleared mainly by the kidney (6) while the whole N terminus appears to be also extracted by other tissues (6) such as lung and liver as is known to occur with ANF (27, 28). Clearance from the circulation is also the most probable reason that when immersion ceases, the C terminus (i.e., ANF) returns to normal within an hour while the N terminus and pro-ANF-31–67 derived from the N terminus are still significantly elevated at 1-hr postimmersion. This is similar to what has been observed with rapid paced heart rates (i.e., another stimulus to the release of the N terminus and the C terminus of the ANF prohormone) (11). With ventricular cardiac pacing at heart rates of 125 beats/min and above, both the C terminus and the N terminus of the prohormone are released, but with cessation of pacing the C terminus (ANF) returns to prepacing levels within 30 min while the N terminus is still elevated at 2 hr postpacing, although steadily declining from the time the pacing was stopped (11). Thus, both in pacing and in water immersion-induced central hypervolemia, the prolonged elevation of the N terminus with cessation of stimuli probably reflects its longer half-life in the circulation. This prolonged half-life of the N terminus may also help explain the persistent natriuresis seen after head-out water immersion when the circulating concentration of ANF has returned to its "normal" preimmersion levels.

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