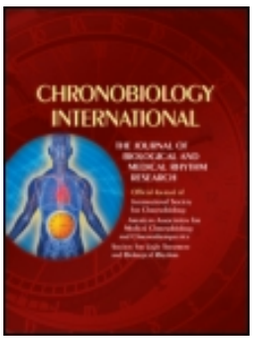

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THE CIRCADIAN RHYTHM OF THE N-TERMINUS AND C-TERMINUS OF THE ATRIAL NATRIURETIC FACTOR PROHORMONE

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Abstract—Circadian variation in the circulating concentrations of the N-terminal and C-terminal portions of the atrial natriuretic factor prohormone (pro ANF) was evaluated in 8 men, ages 41–47, who have been followed for 19 years with respect to circadian variation in physiological variables including blood pressure and clinical chemistries. The N-terminus of the ANF prohormone contains two peptides consisting of amino acids 1–30 and 31–67 while the C-terminus contains 1 peptide (amino acids 99–126) of this 126 amino acid prohormone which lower blood pressure and have natriuretic properties. To determine if either the N-terminus and/or the C-terminus of the prohormone have a circadian variation in their circulating plasma concentrations these 8 men had blood samples obtained for radiommunoassay every 3 hr during a 24-hr period. Three radiommunoassays which immunologically recognize (1) the whole N-terminus (i.e. amino acids 1–98), (2) the midportion of the N-terminus (amino acids 31–67) and (3) the C-terminus (amino acids 99–126) of the ANF prohormone were utilized. The whole N-terminus, the midportion of the N-terminus which circulates after being proteolytically cleaved from the rest of the N-terminus, and the C-terminus each had a peak circulating concentration between 0400 and 0700 which were significantly ($P < 0.001$) higher than their concentrations at any other time throughout the 24-hr period. It was concluded that there is a circadian rhythm in both the N-terminus and C-terminus of the ANF prohormone with peak plasma concentrations near the 24-hr nadir in blood pressure suggesting a possible cause for this blood pressure nadir since both the N-terminus and C-terminus of this prohormone contain blood pressure lowering peptides.

Key words—Atrial natriuretic factor, blood pressure, N-terminal prohormone peptides

Introduction

Circadian variation in physiological variables including blood pressure and clinical chemistries have been followed longitudinally in 13 young solidiers evaluated first while in their mid-20's in the spring of 1969 (1–9). In the spring of 1979, 7 of these same men had their 10 year-replicated circadian profiles documented (10–15). Eight of the original 13 subjects were recently re-evaluated 19 years after their first evaluation. In the 1980's after the first 2 studies were completed, new peptide hormones originally isolated from the atrium of the heart were discovered that lower blood pressure and have natriuretic and diuretic properties (16–18). The evaluation of a possible circadian variation of these natriuretic hormones was incorporated into the study design of the 19 year follow-up of the above subjects and is the basis of this report.

The observation that atrial cardiac extracts could cause natriuresis and diuresis was first made by DeBold and colleagues in 1981 when they infused the supernatants of rat cardiac atria and ventricles into other rats and found that the rat atria but not the extracts from rat ventricles

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caused a dramatic diuresis and natriuresis with urine flow increasing 10-fold and sodium excretion increasing 30-fold (16). Since 1981, progress on the identification and actions of the peptides from cardiac atria have been truly remarkable. It is now known that the peptides which cause this natriuresis have a prepro-hormone of 151 (human, dog, cow) or 152 (rat, mouse) amino acids which lose their hydrophobic leader sequences to form a 126 amino acid prohormone (19–22). This prohormone is the primary form in which the atrial peptides are stored in the perinuclear granules of atrial myocytes (19–22).

All 126 amino acids of this atrial natriuretic factor prohormone (pro ANF) are released into the circulation in man in response to various stimuli (23–30). Thus, the N-terminus consisting of amino acids 1–98 as well as the C-terminus consisting of amino acids 99–126 (which is also called atrial natriuretic factor, ANF) of this prohormone circulate normally in humans (23–30). In addition, a 3900 mol. wt peptide from the midportion of the 98 amino acid N-terminus consistent with amino acids 31–67 (i.e. pro ANF 31–67) also circulates as a separate peptide in man and animals and increases secondary to the same stimuli which increase the whole N-terminus and the C-terminus of this prohormone in the circulation (27–30). Since the midportion of the 98 amino acid N-terminus of the ANF prohormone circulates and the whole N-terminus circulates, this suggests that peptides on either side of amino acids 31–67 also circulate after being proteolytically cleaved from the prohormone (27). Thus, a peptide consisting of amino acids 1–30, i.e. pro ANF 1–30, and a peptide consisting of amino acids 68–98 or smaller peptides derived from these 2 amino acid segments most likely also circulate, although this has not been documented at present (27). The fact that N-terminus and pro ANF 31–67 have been demonstrated to circulate becomes important in light of the fact that pro ANF 31–67 and pro ANF 1–30 have diuretic (18), natriuretic (18), and vasodilatory properties (17) similar to ANF. These peptides as well as ANF at the cellular level involve enhancement of particulate guanylate cyclase with resultant increase in the intracellular messenger cyclic GMP (17,31) as part of their mechanism of action.

Subjects, Materials and Methods

Eight men, 41–47 years of age, who served as subjects in our 14–15 May 1969 study conducted at 4th Army Medical Laboratory, Fort Sam Houston, San Antonio, Texas (latitude: 29.25N, 98.30W), served again as subjects in our 18–19 May 1979 and May 8–9, 1988 studies, conducted at the Special Diagnostic and Therapeutic Unit of the Veterans Administration Hospital, Hines, IL (latitude: 41.49N,87.37W). The protocols followed in both studies were similar if not identical, as were the analytical procedures employed (1, 15).

All subjects were administratively admitted to two hospital wards of the Special Diagnostic Unit, immediately after leaving their daily routines of civilian life. No special standardization to the hospital environment was made. Each subject was given a physical examination by the physician member of our staff. Height and body weight were recorded. Participants were regularly diurnally active, taking sleep at night. The protocol of the study, familiar from earlier studies, was reviewed briefly with the subjects. Lights were turned off at 2215 and on at 0615. Meals consisted of a general hospital diet totalling 2500 calories and were served at 0730, 1330 and 1630. The food was prepared and served by the dietetic service of the hospital. The food actually consumed was monitored for each subject and any item not eaten was subtracted from the intended dietary intake. The average calories consumed by all subjects were 2250 and ranged between 1834 and 2516. The subjects were not restricted in their water intake, except for the half hour prior to sampling, but were required to abstain from other liquids and food between meals. Nutritional assessments, including anthropometric measurements, were made for each subject; all were found to have a good nutritional status.

Blood samples were collected at 3-hr intervals beginning at 1900 on 8 May with subsequent sampling at 2200, 0100, 0400, 0700, 1000, 1300 and 1600. The sequence of sampling involved the

following, in this order: heart rate, oral temperature, blood pressure, intraocular pressure, and blood sampling. Blood obtained was centrifuged, serum separated and aliquots for assays were immediately frozen at -25°C until analsis.

Radioimmunoassays for pro 1-98, pro ANF 31-67 and ANF

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 previously described by our laboratory (27,30). Our pro ANF 1-30 radioimmunoassay recognizes a component in plasma of approximately 10,000 mol.wt as characterized by G-50 Sephadex gel-permeation chromatography which is consistent with the whole N-terminus of the ANF prohormone (i.e. amino acids 1-98), but without the C-terminus attached to it (27). The pro ANF 31-67 radiommunoassay recognizes in plasma a component of the N-terminus of approx 3900 mol.wt which is consistent with measuring only amino acids 31-67 of the prohormone (i.e. pro ANF 31-67, mol.wt 3878). Our ANF radiommunoassay recognizes a 3000 mol.wt peptide in plasma with ANF's actual mol.wt being 3081. All determinations were performed in triplicate. The interassay coefficient of variation for pro ANFs 1-30, 31-67, and ANF radiommunoassays were 4.8%, 5.3%, and 5.7% respectively. The interassay coefficient of variation was 8 percent for both pro ANFs 1-30 and 31-67 radioimmunoassays while ANF's radioimmunoassay interassay variation was 6.9%.

Recovery was examined by adding synthetic unlabelled pro ANF 1-30, pro ANF 31-67 and ANF at 100, 200, and 400 pg/ml to pooled plasma. Recovery of pro ANF 1-30 was 83.5 ± 13.2 (S.D.)% while pro ANF 31-67 recovery was 100.9 ± 8.9%. Recovery of ANF was 92 ± 11%. The respective IC_{50}'s were 180, 120 and 11 fmols/tube while the lowest detectable concentrations were 40, 35 and 1.4 fmols for pro ANFs 1-30, 31-67 and ANF radioimmunoassays, respectively. Serial dilution of pooled plasma has revealed excellent parallelism of standard and unknown in these assays (27, 30). Reverse phase high pressure liquid chromatography utilizing Novapak C-18 (5 micron) cartridge columns revealed that the pro ANFs and ANF measured were authentic.

Statistical analysis

The data obtained by the above immunoreative (ir) radioimmunoassays were illustrated as the group mean ±S.E.M. at each time point. All data were analyzed for time-effect by the one way analysis of variance (ANOVA) and for circadian rhythm by a computerized inferential statistical method involving the fit of a 24-hr cosine curve to individual data series by the method of least squares. A *P*-value for the rejection of the zero circadian amplitude assumption, the acrophase (timing), amplitude and the MESOR were determined on each individual data series (32). Group results were summarized by population mean cosinor (33).

Results

Each subject showed a large range of change (ROC) from lowest to highest value in each pro ANF segment over the 24-hr observation span. The group average ROC for pro ANF 1-98 was 107%, for pro ANF 31-67 it was 115% and for the C-terminus (ANF; 99-126) it was 76%. The mean serum concentrations of the whole N-terminus (i.e. amino acid 1-98; pro ANF 1-98) and pro ANF 31-67 (i.e. amino acid 31-67) from the midportion of the N-terminus of the ANF prohormone measured every 3 hr during a 24 hr span are illustrated in Figures 1 and 2, respectively. As observed in these figures there was a marked increase in the whole N-terminus and pro ANF 31-67 at 0400. This elevation of the N-terminus and the midportion of the N-terminus remained elevated at 0700 and then decreased by one-half at 1000 to circulating concentrations that thereafter remained fairly stable the rest of the day. The circulating concentrations of the whole N-terminus and pro ANF 31-67 at 0400 and 0700 were significantly increased ($P < 0.001$) compared to all other time points throughout the 24 hr period.

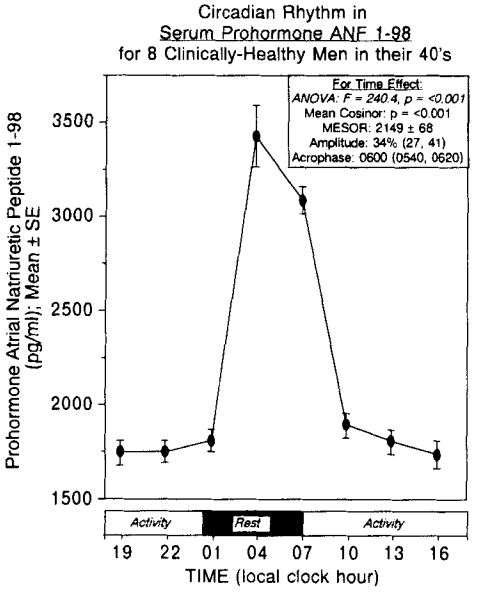

Figure 1. Mean plasma concentrations (± S.E.M.) of the whole N-terminus (i.e. amino acids 1–98, i.e. pro ANF 1–98) of the atrial natriuretic factor prohormone in 8 healthy men in a 24-hr period of normal activity. In this evaluation in May of 1988 each volunteer consumed a total of approx 2500 calories in meals taken at 0730, 1330, and 1640. Lights were turned off at 2215 and on at 0615. The peak values at 0400 and 0700 of pro ANFs 1–98 were significant at $P < 0.001$ compared to all other time points for each respective peptide by cosinor analysis.

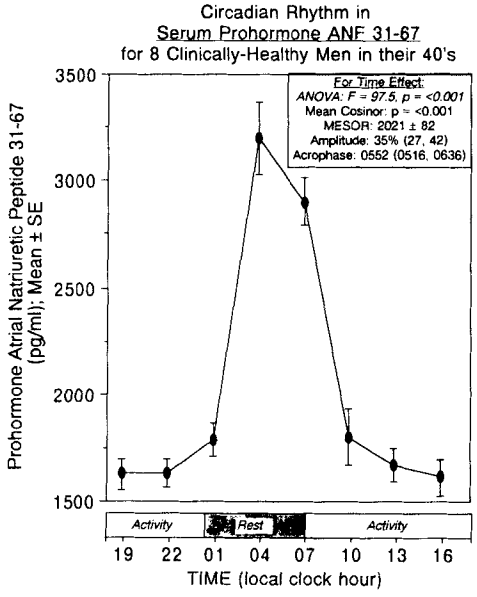

Figure 2. Mean plasma concentrations of the midportion of the N-terminus (amino acids 31–67, i.e. pro ANF 31–67) of atrial natriuretic factor prohormone in 8 healthy volunteers in a 24-hr period of normal activity. The peak values at 0400 and 0700 of ANF were significant, $P < 0.001$ compared to all other time points by cosinor analysis.

Atrial natriuretic factor, the C-terminus of this 126 amino acid prohormone, which consists of amino acids 99–126 followed a similar pattern (Figure 3). Thus, in these eight healthy volunteers atrial natriuretic factor was nearly double at 0400 its concentration at 2200. The circulating concentration of ANF was still markedly increased at 0100 and then decreased by 1000 to its circulating concentration in these individuals observed at 2200. Thus, both the N-terminus and the C-terminus of the ANF prohormone increased at 0400, remained elevated at 0700, and then decreased back at 1000 to circulating concentrations similar to those observed at 2200. The high-amplitude circadian rhythms of both the N-terminus and C-terminus of ANF prohormone were highly significant by both analysis of variance and cosinor techniques as observed in Table 1 and Figures 1, 2 and 3.

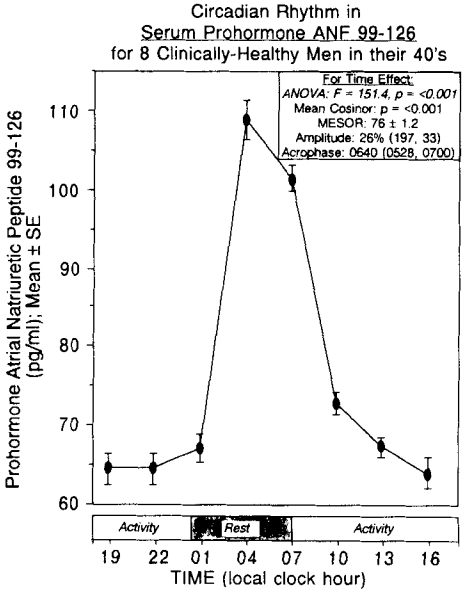

Figure 3. Mean plasma concentrations of the C-terminus (amino acids 99–126 i.e. ANF) of atrial natriuretic factor prohormone in 8 healthy volunteers in a 24-hr period of normal activity. The peak values at 0400 and 0700 of ANF were significant $P<0.001$ compared to all other time points by cosinor analysis.

Table 1. Cosinor analysis of the circulating concentration (pg/ml) of the whole N-terminus (Pro ANF 1–98, the midportion of N-terminus (Pro ANF 31–67) and the C-terminus (ANF) that validates a circadian rhythm for both the N-terminus and C-terminus of the atrial natriuretic factor prohormone.

	P	Mesor pg/ml	S.E.	Amplitude	(95% Limits)	Acrophase*	(95% limits)
pro ANF 1–98	0.001	2149	68	720	(563,878)	05^{55}	(05^{40},06^{20})
pro ANF 31–67	0.001	2021	82	696	(537,856)	05^{52}	(05^{14},06^{36})
ANF	0.001	76.1	1.2	19.8	(15.2,24.3)	06^{40}	(05^{28},07^{00})

*Acrophase in clock hour and minute; reference = local midnight = 0000.

Discussion

The present investigation demonstrates that the whole N-terminus, the midportion of the N-terminus (i.e. pro ANF 31–67), and the C-terminus (i.e. atrial natriuretic factor, ANF) of the ANF prohormone each have similar circadian rhythms with an approximate doubling of these peptides at 0400 compared 2200. The present study combined with the one previous study by one of us (DV) (24) which evaluated the circadian rhythm of the circulating concentrations of the N-terminus, pro ANF 31–67, and the C-terminus at 4-hr intervals in house staff helps to accurately define the circadian rhythm in healthy adults because of the overlapping time points utilized in the 2 studies. In the previous study (24) at the time point 2400 there was no statistical difference from the rest of the day while at 0400 there was a similar increase to that found in the present investigation. Thus, these peptides begin to increase between 0100 and 0400, remain elevated through 0700, and then decrease to approx their 2200 concentration by 0800. Although the present study involved only men who had been in the military together, in the previous study of these peptides both men and women were evaluated and they had identical rhythms (24). The nearly identical circadian variation of the present investigation performed in the spring with the previous investigation of the N-terminus and C-terminus of the ANF prohormone being performed in the fall suggests that there is no seasonal variation in humans in the timing of their circadian rhythms of the ANF prohormone. Atrial natriuretic factor's rhythm, as pointed out by Halberg *et al.* (34), is similar to cortisol in its anticipatory circadian periodic rise prior to awakening, preparing for each day's activity. The same relationship to cortisol is true for the N-terminus of the ANF prohormone as observed in the present investigation.

There have been no other investigations of the N-terminus of the ANF prohormone with respect to circadian variation, but there have been 2 other studies with respect to ANF and circadian variation (35,36). In the study by Donckier *et al.* (35) identical results to the present investigation were found with the ANF peak concentration being at 0400. The one other study (36) kept 7 volunteers recumbent for 24 hr and found only a very slight variation of ANF over the 24 hr period with a "peak" at 1100 or 1300 that was actually only a couple pg/ml different from the values at 0800, 1600, 2400 or 0400 (36). Recumbancy for 24 hr, thus, does appear to affect the circadian peak of ANF that is seen in healthy adults adhering to a more normal pattern of working during the day and sleeping at night.

With respect to blood pressure and these newly discovered blood pressure lowering peptides from the heart, mean arterial pressure has been shown to correlate with atrial natriuretic factor in a longitudinal study of ANF and blood pressure over a 12 week period (37). The N-terminus of the prohormone containing the vasodilatory peptides pro ANF 1–30 and pro ANF 31–67, likewise, correlates closely with mean arterial pressure (McMurray and Vesely, unpublished observation). Infusion of ANF has also been shown to lower blood pressure in normotensive and hypertensive individuals (38, 39). The findings of the present investigation that the circulating concentration of the N-terminus and the C-terminus of the ANF prohormone peak near the normal 24 hour nadir of blood pressure (40,41) may help to explain why blood pressure is lowest at this time of the day.

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